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Contents

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From the Editor

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Rhododendrons International (*RI)* is an online journal distributed free to all the world's known rhododendron associations for their internal distribution. It can also be accessed without charge on the American Rhododendron Society website at https://www.rhododendron.org/ ri-index.htm. This third volume of *RI,* broken into two parts because of the large size of the articles, contains the first series of research articles from the Rhododendron Research Network (R-RN), which is a collaboration between the American Rhododendron Society (ARS) and an international group of rhododendron researchers. More detail about the objectives of this network and what is contained in this volume are given in the introductory chapter by the network's co-chairs.

This network is an exciting collaboration between the ARS and the world's rhododendron researchers, and we hope that other rhododendron societies will also actively engage in this initiative by widely sharing this volume with botanists in their own countries and encouraging them to also become participants. I look forward in the future to receiving more dedicated research articles and producing another RI volume to further this endeavour.

The Unique Value of Genus *Rhododendron* for Investigating the Evolutionary Ecology of Root-microbe Interactions

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Introduction

Rhododendrons are quite famous for their diverse leaf forms and floral displays, but much less is known about how root characteristics might vary across the genus. Here we describe some physiological, ecological and evolutionary aspects of roots, and we relate this to recent work showing that the genus *Rhododendron* has a high root diversity. We discuss the relative influences of evolutionary history and current climate on root traits, and the fundamental differences across deciduous and evergreen *Rhododendron* clades in their reliance on improved foraging scale (i.e., exploring a larger area) versus foraging precision (i.e., intensively exploiting a smaller area) to manifest rapid resource uptake. In addition, we highlight the close associations between *Rhododendron* roots and a wide diversity of soil microbes, which can help or hinder plant growth, and which likely influence *Rhododendron* root morphology, species climate preferences and niche partitioning of co-occurring species. Finally, we suggest that this multi-dimensional diversity within *Rhododendron* has the potential to provide unique insight into important, big-picture questions concerning the evolutionary ecology of roots in general and their associated microbes.

The functional and ecological aspects of roots and their associated microbes

Root systems facilitate the acquisition of nutrients and water from soil, stabilize the

soil matrix, and anchor plants to a fixed position in the soil (Den Herder *et al.* 2010). Root traits are closely tied to plant performance (Reich *et al.* 1998) and environmental factors like water availability (Larson and Funk 2016), so root function likely influences the plant niche, or the set of conditions where a population can persist (Figure 1). Root diversity across climate gradients is often cited as evidence that root traits can evolve, and that they represent important adaptations to climate. For example, cold temperatures are stressful, and roots from colder climates show evidence of increased stress tolerance compared to species from warmer climates (Soudzilovskaia *et al.* 2015, Medeiros *et al.* 2017, Valverde-Barrantes *et al.* 2017). More generally, climate gradients can be thought of as a niche axis, and different root strategies might enhance performance in different habitats. Even within a single plant community, there are diverse root morphologies, such as both deep and shallow-rooted species (Lynch 1995), and diverse root functions could enhance co-occurrence in plant communities (Nobel 1997), essentially allowing plants to partition the niche.

The morphology of roots strongly influences their ability to acquire nutrients and water under different environmental conditions, which has been demonstrated in a variety of plants (Lyu *et al.* 2016). The surface of the root is where resource uptake occurs, so when resources are limited, plants require more root surface area to acquire adequate water or nutrients (Luo *et al.* 2013). Quantitatively, root surface area is characterized by root length, diameter and branchy-ness. Longer, thinner roots have more surface area than shorter, fatter roots. Also, long, unbranched roots allow plants to explore farther afield, increasing the scale of resource foraging. Surface area can also be
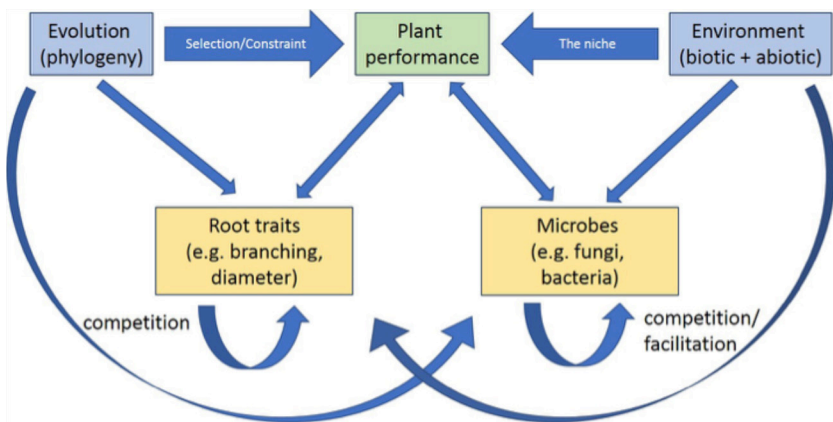

Fig. 1. Conceptual diagram outlining in an evolutionary ecology context the interactions among *Rhododendron,* soil abiotic properties, and the soil microbial community.

increased by adding more root branches, and more branchy root systems can proliferate rapidly within a small soil volume to effectively monopolize resources, which makes them good at precision exploitation of nutrient hot-spots (Campbell *et al.* 1991). In addition, specific root length (SRL, root length per carbon investment) relates strongly to root resource acquisition, life-span and stress resistance. Compared to those with low SRL, species with higher SRL exhibit more rapid resource uptake rates (Reich *et al.* 1998), but their roots have a shorter lifespan (McCormack *et al.* 2012) and lower stress resistance (Ostonen *et al.* 2007). Lastly, even within a species, all of these morphological features can change in response to environmental conditions such as soil type and water availability (Bengough *et al.* 2011) or nutrient availability (Dai *et al.* 2012).

The high diversity of root morphology across environments and among the world's plant species speaks to the possibility that root traits may also evolve in response to the environment (Bingham and Bengough 2003, Bengough *et al.* 2011). Importantly, the past evolutionary history of plant species ("phylogeny") also shapes the diversity of root traits we observe today. In a comparison across 34 species, Valverde-Barrantes *et al.* (2015) found that closely related woody plants are more similar to one another in their roots than to more distantly related plants. Thus, in some cases, root traits could represent a constraint imposed by past selection rather than an adaptation to current climate. How then might traits persist within lineages without providing a current fitness advantage? Past selective sweeps could reduce trait variation, and if this is followed by a period of relaxed selection, i.e., a reduction in the strength of selection on the trait (Lahti *et al.* 2009), then the conditions in which we find a particular root type today may not reflect the same selective forces which shaped that trait over evolutionary time. As long as selection is relaxed, and the trait is not too energetically expensive compared to alternative trait expressions, then an unhelpful trait may persist under the new conditions. This means that care must be taken to account for evolutionary history in the study of any plant traits or characteristics.

Understanding the relative roles of phylogeny and climate in driving the evolution of root morphology should continue to be a fruitful avenue of investigation, but we cannot overlook the potentially important role of sympatric soil microbes, which often interact directly with roots to affect root foraging functions (Chen *et al.* 2016) and plant growth (Pieterse *et al.* 2014; Figure 1). On the one hand, some microbes may have negative and harmful effects on plants. For example, microbial pathogens can impede nutrient uptake, and eventually can cause death (Berg *et al.* 2009). On the other hand, other microbes can be beneficial to plants by facilitating nutrient uptake from soil, or even improving resistance against soil microbial pathogens (Baral *et al.* 2013, Berg et al. 2009, Den Herder *et al.* 2010, Downie *et al.* 2015). The most famous of the beneficial soil microbes are the mycorrhizae.

The origin of mycorrhizae can be traced back to 400 million years ago (Remy *et al.* 1994), and today the mycorrhizal symbiosis between fungi and plants is prevalent. Mycorrhizae have been found to associate with nearly 80% of land plants (Wang and Qiu 2006), and through close associations with root cells, mycorrhizae provide nutrients to the plant (e.g., nitrogen and phosphorus; Marschner and Dell 1994) while in return, plants provide sugar to fuel fungal growth (Hobbie and Hobbie 2006). Arbuscular mycorrhiza is the most predominant association, in which fungi penetrate the root cells of their host plants through hyphae or arbuscules (Pfeffer *et al.* 1999, Schüßler et al. 2001, Wang and Qiu 2006). Unlike arbuscular mycorrhiza, ectomycorrhizal fungi do not penetrate plant cells, but instead form a hyphae network called the Hartig net, which can be found in the root's intercellular spaces (Blasius *et al.* 1986, Tedersoo *et al.* 2010). Ericoid mycorrhiza are another important and special type of mycorrhiza found only in the plant family Ericaceae. Ericoid mycorrhizal fungi just penetrate the cell walls but not the plasma membrane, and their hyphae form a loose pattern (Read 1996). It is reported that ericoid mycorrhiza help Ericaceous plants colonize in acidic or soil polluted with toxic metals, which may have played a critical role in the evolutionary adaptations to harsh habitat environments within family Ericaceae (Cairney *et al.* 2003).

Together, roots and root-associated microbial communities are indispensable for their unique ecological effects, playing an essential role in driving nutrient cycling in terrestrial ecosystems (Berg *et al.* 2009) and in shaping biological communities. A large proportion of total carbon products produced by plant photosynthesis is allocated to roots, and both roots and mycorrhizal fungi use it (Hobbie and Hobbie 2006). Through their effects on plant growth and decomposition, soil microbes, and especially mycorrhizal fungi, play an important role in controlling the carbon flow between the atmosphere and soils (Simard and Austin 2010). For example, when there is an increase in soil carbon availability, some high biomass fungi may dominate the microbe community, altering the soil carbon balance and limiting access to carbon, rather like a gatekeeper (Treseder 2005, Hobbie and Hobbie 2006).

It is also important to realize that soil microbes are not randomly co-occurring (Saari *et al.* 2005). Just like plants, the diversity and function of the microbial community can be shaped by evolutionary history, as well as by current abiotic and biotic factors (Berg *et al.* 2009). Abiotic factors represent all the environmental conditions that plants experience, such as soil type, geographic location, climate patterns, or even the use of pesticides or antiseptics (Berg *et al.* 2009). Biotic effects on root communities can include human activities like nutrient deposition (Naples and Fisk 2010), climate change and climate variability (Tylianakis *et al.* 2008) or a change in biodiversity, such as through the introduction of exotic species (Kourtev *et al.* 2002). Interactions between

the microbes themselves, e.g. as facilitation or competition, can also play an important role in shaping the microbe community (Gorzelak *et al.* 2012). Microbial communities also respond to the direct consequences of plant community biodiversity (Burns *et al.* 2015), plant stage of development (Tingey *et al.* 2000), plant health (Purmale *et al.* 2012), or the composition of root exudates (Berg *et al.* 2009). In fact, moving from the surrounding soil toward the root surface, the composition of the microbial community becomes increasingly more specific, and different plant species may support unique microbial communities (Berg et al. 2009). Importantly, mycorrhizal fungi vary widely in their specificity, ranging from the most generalist arbuscular mycorrhiza fungi to the highly host-specific ericoid mycorrhizae.

Rhododendron, a study system with multi-dimensional diversity

The 1000+ species of *Rhododendron* are broadly distributed across ecological and latitudinal gradients (Cox and Cox 1997) from the Arctic to the tropics. In terms of the soil environment, however, they have often been viewed as uniform, as most rhododendrons thrive on soils with a relatively low pH and low fertility (Wurzburger and Hendrick 2006). In addition, they are Ericaceous plants, which means that they host ericoid mycorrhizae on their roots (Read 1996). The importance and abundance of mycorrhizal fungi should be greater for species adapted to low fertility sites, because mycorrhizal fungi are exceptionally efficient at nutrient uptake (Marschner and Dell 1994). However, few studies have addressed root traits in Ericaceae lineages, perhaps because their strong association with specialized soil types and the presence of ericoid mycorrhizae suggests that their root traits might have low variation (Read 1996).

When Medeiros *et al.* (2017) examined 27 species of *Rhododendron* from a variety of habitats, they unexpectedly found a high diversity of root morphology across the genus. The potential influence of evolutionary history was then investigated using phylogenetic comparative analysis across four taxonomic sections: *Pentanthera* (deciduous azaleas)*, Ponticum* (elepidotes)*, Rhododendron* (lepidotes), and *Tsutsusi* (evergreen azaleas). This analysis showed that close relatives were strongly similar in the leaf trait of specific leaf area (leaf area per carbon investment), but were not similar in their mean root traits (Figure 2). Compared to species in section *Ponticum*, within section *Pentanthera,* specific root length (SRL) was more variable among species and across sites, but *Pentanthera* had more root tips per gram of carbon invested (higher specific root tip abundance). Furthermore, the variation observed in SRL across these rhododendrons represents >50% of global variation in this trait (Valverde-Barrantes *et al.* 2017). Thus, species within the genus *Rhododendron* are exceptionally diverse in their root morphology, especially after accounting for evolutionary history (Medeiros *et al.* 2017), suggesting that the root traits of rhododendrons have experienced a highly diversifying
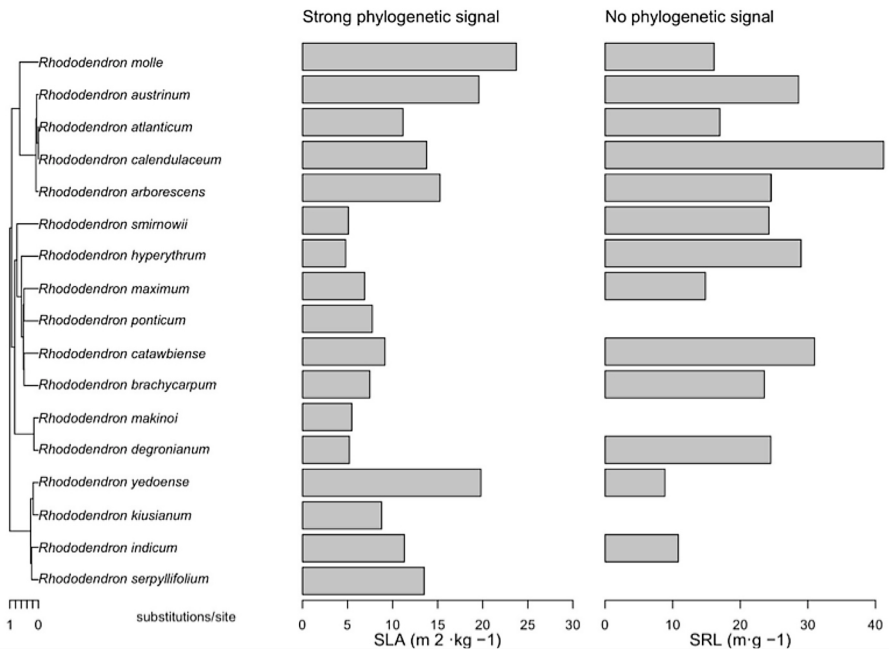
Fig. 2. The effect of phylogenetic relationships on leaf and root traits of rhododendrons based on data presented in Medeiros et al. (2017). The phylogenetic relationships among species were estimated using DNA sequences obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Among the studied species, specific leaf area (SLA) exhibited a strong phylogenetic signal, such that closely related species were more similar than expected by chance. Conversely, specific root length (SRL) was not similar among close relatives. The evolutionary distance between species is shown as the number of DNA nucleotide substitutions per site.

evolutionary history. These observations lead to the question: Is there evidence that *Rhododendron* root traits represent specific adaptations to climate variability? Medeiros *et al.* (2017) investigated the potential role of climate by comparing roots of species originating from either warm or cold climates, and by examining plants growing in two different botanical garden climates (Figure 3). Within a species, root traits differed strongly between botanical garden climates, indicating that rhododendrons have a strong capacity to adjust their root traits in response to environmental cues. Species originating from warm climates had different root traits from those from colder climates, particularly for species in sections *Pentanthera* and *Rhododendron*. Combined with the lack of evidence for evolutionary constraints on mean root traits, this result is consistent with the idea that *Rhododendron* root traits represent adaptations to climate.

Perhaps the most fascinating evidence that root diversity represents adaptations

Figure 3. Root samples examined by Medeiros *et al.* (2017) were collected in 2014 at The Holden Arboretum Layer Rhododendron Garden (top left) and in 2015 at the Rhododendron Species Foundation Botanical Garden (bottom left). Excavations uncovered wide variation in *Rhododendron* root systems, for example, a large, highly branched root system collected from beneath *R. triflorum* (top right) and a smaller, less branched root system collected beneath *R. calendulaceum* (bottom right).

to different climates is that *Rhododendron* species exhibit two distinct root foraging strategies. For species originating from or growing in warmer climates, their roots should have higher nutrient and water uptake rates compared to those from colder climates, because more active roots are needed to support the higher growth rates typical of warm climates (Reich 2014). Higher uptake rates can be accomplished by increasing root surface area per unit carbon invested, but there are two ways to do so. For a given unit of carbon invested, species within section *Rhododendron* (i.e., lepidotes) increased their total root length, while those in section *Pentanthera* (i.e., deciduous azaleas) increase the number of root tips (Figure 4, Medeiros *et al.* 2017). These two strategies have very different ecological implications. An increase in root length per unit carbon invested increases the scale of root foraging, allowing plants to search farther afield for nutrients. Thus, species with a large foraging scale should be more adept in environments that present consistently low nutrient availability across the landscape, but which have a
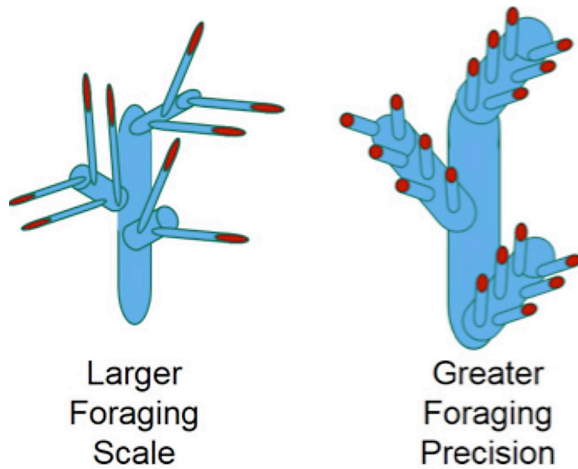
Larger
Foraging
Scale

Greater
Foraging
Precision

Figure 4. Two types of root morphology were observed in rhododendrons, based on data presented in Medeiros et al. (2017). As more resources are needed by a plant, some species build more root length per gram of carbon invested (left), which translates to a larger foraging range. In contrast, other species make more root tips per gram of carbon invested (right), which results in more intense foraging capacity in a given area.

low plant density, so that exploratory roots do not face strong competition from other species. In contrast, an increase in the number of root tips per carbon invested will improve the precision and intensity of root foraging, such that the plant can more effectively monopolize resources and take advantage of ephemeral nutrient hot-spots. These species should have an advantage in pulse-driven ecosystems, where high levels of competition limit the scale of foraging.

Though climate likely has a role to play in promoting root diversity in rhododendrons, Medeiros *et al.* (2017) found that root traits within section *Ponticum* (elepidotes) are only weakly related to climate. Given what we know about the importance of microbes for root function, could microbial community also drive the evolution of root traits within rhododendrons? The microbes associating with the roots of rhododendrons are many and vary greatly, and they include mutualists like mycorrhizal fungi, as well as pathogens and microbes with unknown functional attributes. *Rhododendron* roots are very commonly associated with certain ericoid mycorrhizal fungi such as *Oidiodendron* (Bougoura and Cairney 2005, Grunewaldt-Stöcker *et al.* 2013, Parker 2013, Sun *et al.* 2012, Tian *et al.* 2011, Vohnik *et al.* 2005, Zhang *et al.* 2009), *Rhizoscyphys* (Bougoura and Cairney 2005, Sun *et al.* 2012, Tian *et al.* 2011, Vohnik and Albrechtova 2011) and *Helotiales* (Bougoura and Cairney 2005, Sun *et al.* 2012, Tian *et al.* 2011). In addition, several studies have demonstrated colonization of *Rhododendron* roots by

ectomycorrhizae (Parker 2013, Sun *et al.* 2012, Vohnik *et al.* 2007) and AM fungi (Chaurasia *et al.* 2005). Bacteria with anti-microbial activity have also been observed on *Rhododendron* roots (Baral *et al.* 2011, Shimizu *et al.* 2000), but various pathogenic microbes have also been found on *Rhododendron* roots (Farr 1996, Linderman 1986), including the oomycete *Phytophthora* which has been named as one of the most influential and dangerous invasive species on Earth (Lowe *et al.* 2000, Nentwig *et al.* 2017). Just as intriguing, a number of microbial groups commonly observed on *Rhododendron* roots have unknown function, such as some bacteria (Grunewaldt-Stocker *et al.* 2013, Parker 2013) and the dark septate endophytes (Bougoura and Cairney 2005, Parker 2013, Sun *et al.* 2012, Vohnik and Albrechtova 2011, Vohnik *et al.* 2005). The role of the complex and multifaceted microbiome in *Rhododendron* evolution, including root trait evolution, is thus far largely unexplored and not well understood.

Interestingly, there is some evidence that soil microbes alter the response of *Rhododendron* root traits to climate. In the wild, the composition of ericoid rhizosphere microbes is closely related to elevation (Gorzelak *et al.* 2012). Within the ericoid genus *Vaccinium*, which is closely related to *Rhododendron*, the rhizosphere microbe community in alpine habitats of east-central British Columbia is dominated by *Rhizoscyphus ericae*, while at the bottom of low elevation valleys, *Phialocephala fortinii* was the dominant microbe species (Gorzelak *et al.* 2012). The general diversity of the *Rhododendron* rhizosphere microbe community also decreases moving upward from 1500m to 4500m elevation (Chaurasia *et al.* 2005), similar to the well-described pattern where plant diversity decreases with increasing elevation. Finally, abiotic factors are often correlated with soil microbes, for example, differences in soil pH influence the distribution of soil fungi. In alpine areas with acidic soil (pH = 4.6), the number of spores from fungi in the roots of rhododendrons was smaller than those in areas with a higher pH (pH = 5.2, Chaurasia *et al.* 2005). Whether or not the morphological traits of rhododendrons change along environmental gradients in concert with these changes in root microbial community is currently unknown.

Looking to the future - what can we learn from rhododendrons?

The multi-dimensional diversity within the genus *Rhododendron*, including its diverse taxonomy, wide geographic range, multiple climate preferences, variable root morphology, ease of accessibility, and unique microbial associations described here, provide an excellent study system for understanding the evolutionary ecology of root-microbe associations. Here, we highlight four "big-picture questions" for which *Rhododendron* represents a first-class study system:

1. How does root morphology relate to plant-microbe interactions?

Plants often respond to microbes in the soil near their roots by altering root traits, but the relationships among plant traits, soil microbes, and plant fitness are not fully understood (Friesen *et al.* 2011). While plant leaf traits such as leaf thickness and plant phylogeny correlate with leaf microbial community composition (Kembel *et al.* 2014), it is not known if similar patterns are true for root associated microbes and root morphology. Root morphology includes patterns in root branching, relative thickness of roots, and root-to-shoot ratios. Root branching might be associated with soil microbe communities if branching patterns affect the volume of soil influenced by a plant. Although the majority of plant functional trait studies have focused on leaf traits, newer work suggests that root traits face fundamentally different selective pressures (Comas *et al.* 2012, Soudzilovskaia *et al.* 2015, Chen *et al.* 2016, Medeiros *et al.* 2017). Thus, studies of root traits and root-microbe associations are likely to generate unique insights into plant functional ecology.

2. How do environmental and evolutionary histories shape root traits?

The study of plant traits holds considerable promise in ecology for providing synthesis and generality in a world that sometimes seems idiosyncratic and species-specific (Wright *et al.* 2004, Shipley *et al.* 2016). Comparative studies within clades of plants are particularly important for understanding the role of evolutionary history in these relationships. For example, rhododendrons with thinner leaves (higher specific leaf area) are associated with environments with warmer temperatures (Medeiros *et al.* 2017). Because closely related *Rhododendron* species are similar in this leaf trait, studies that do not consider evolutionary relationships (i.e., phylogeny) might miss this pattern (Medeiros *et al.* 2017).

Trait-based ecology currently lacks sufficient tests for relationships between functional traits and ecological gradients (Shipley *et al.* 2016). The remarkable global range of rhododendrons (many northern hemisphere habitats) crosses many ecological and altitudinal gradients, making this genus a strong candidate for comparative studies of plant traits. Because rhododendrons are often found in mountainous regions, and because species composition and the phenology of rhododendrons often shifts along elevation gradients (Hart *et al.* 2014), studies across elevation gradients are particularly promising. For example, functional traits like root morphology are well-known to vary across multiple continents, but within *Rhododendron* one could ask whether they correlate with continental and elevation gradients in a similar way, providing a necessary test of the potential generality of these correlations.

Also, by testing for a correlation between root traits and microbial abundance in the field, the assumption that these traits are "functional" can be tested (Shipley *et al.* 2016) to determine which traits directly influence plant fitness, i.e., improved

survival and/or fecundity. In addition, much of the functional trait literature assumes that intraspecific variation is either small, or does not confound our interpretation of functional trait comparisons (Funk *et al.* 2016, Shipley *et al.* 2016). Rhododendron presents a particularly interesting choice for studies that consider the relative influences of non-genetic changes in phenotype in response to environment (plasticity) and genetic differences in determining trait-climate relationships (Medeiros *et al.* 2017).

3. What is the role of the plant root microbiome in plant co-occurrence?

Ecological patterns, such as species co-occurrence patterns, are likely influenced by plant-soil microbe associations. For example, soil pathogens are thought to mediate species co-occurrence in plant communities (Bever *et al.* 2015). The Janzen-Connell hypothesis (Janzen 1970, Connell 1971) suggests that plant community diversity would be enhanced if seedlings performed better growing near other species than near their own species. This pattern could be influenced by species-specific soil pathogens, such as soil fungi, and might minimize the likelihood of a single plant species becoming dominant in a community (Sarmiento *et al.* 2017).

The multi-species complexity of *Rhododendron* communities combined with ecological gradients offer the opportunity to answer questions such as: What are the co-occurrence patterns between microbes and *Rhododendron* species? When different plant species co-occur, do they have similar or divergent soil microbiomes? Is there evidence of plant species-specific soil microbiomes? And particularly for *Rhododendron,* what is the role of evolution in shaping interactions soil pathogens (Bever *et al.* 2015)?

4. What is the role of plant root microbiome in species diversification?

Ecologists and evolutionary biologists have long been interested in understanding the factors driving and constraining diversity. Recent research has begun to explore the possible role of species interactions in species diversification, and interactions between rhododendrons and their soil microbes are a particularly rich area for investigation. For example, in areas where there are many co-occurring species of rhododendrons, could root attributes or soil microbes contribute to niche differentiation and species diversification?

Soil mutualists could influence species diversification over time by allowing plants to colonize otherwise unsuitable habitats (Afkhami *et al.* 2014). This type of facilitation might be very important for ericoid plants, including rhododendrons, because these are often found in lower pH habitats under low nutrient conditions, which is very stressful for many plants. By broadening the set of conditions where these plants can persist (Afkhami et al. 2014), soil mutualists could increase "ecological opportunity" for species diversification (Losos and Mahler 2010). Alternatively, mutualist soil organisms with a limited geographical range could constrain species ranges (e.g., invasion of introduced

pines, Nuñez *et al.* 2009), potentially leading to lower rates of species diversification in taxa that rely heavily on mutualists. Large data sets on *Rhododendron* that combine species interaction data with phylogenies would help address these possibilities.

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invested (right), which results in more intense foraging capacity in a given area.

Floral Morphology of *Rhododendron* and its Relation with Pollinators

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Abstract

Flower diversity within a plant group is directly proportional to the number of pollinator species available to pollinate that plant group, because variations in floral attributes bring about changes in pollinator preference tending to result in more pollination variety, and conversely, changes in pollinator behavior add to floral diversity. A particular set of floral traits can sometimes be studied as a pollination syndrome, a two-way association between that set of traits with its attraction of a specific group of pollinators. *Rhododendron* flowers show sufficient diversity in shape, color, and pigment patterns to warrant a study of pollinator syndromes. To date, there has not been much analysis of ecological and evolutionary aspects of floral attributes i.e. characters and rewards including their value and content, and their collective interaction with pollinators and pollinator groups in this species rich genus.

Here, we analyze information on floral diversity and pollinator types to identify putative pollination syndromes in *Rhododendron*. We do this by coding floral diversity as "character-complexes", or "Cc", which have been looked upon as initial hypotheses of pollination syndromes that we evaluate in the context of known associated pollinators. This approach to understanding the floral biology of this richly diverse genus can be used as a general method to understand the evolution of floral forms and pollinator ecology in other diverse groups of plants.

Introduction

Diversity of floral forms and variation in pollinator behavior are two characteristic features of the dynamics of flower-pollinator interaction that in turn affects reproductive fitness of a species. One of the most significant fitness attributes in plants is success of fertilization by a pollinator. A pollinators' choice of flower is affected by flower pigmentation including presence or absence of pigment patterns (Hansen *et al.* 2012), shape and size of the flower, and size of pigment patterns (Medel *et al.* 2003), symmetry of the flower including orientation (Jesson and Barrett 2003; Citerne *et al.* 2010), type and content of floral reward (Alm *et al.* 1990; Perret *et al.* 2001; Krömer *et al.* 2008), among many other floral attributes. Characterisation of floral morphology is a first step in any floral evolutionary and ecological study in any plant group.

Rhododendron, as a highly diverse and abundant group of species, can serve as a model system for study in varied disciplines of plant science. Its selection as the system to study floral evolution and ecology in woody perennials can address numerous questions and potentially provide answers and understanding that will be relevant to other plant groups.

The main objective of this study is to use recent understanding of the ecological and evolutionary basis of floral diversity in *Rhododendron* to identify putative pollination syndromes in this genus. In a recent study (Berry *et al.* 2018), an evolutionary association was shown in *Rhododendron* between floral monosymmetry [= being divisible into two like parts bilaterally by a single plane through the axis of the flower] and the presence of pigment patterns on the corolla. Subtle differences in flexion of style or stamens, or bundling of stamens can lead to a monosymmetric condition in a flower with a polysymmetric [= divisible into like parts by more than one axial plane] corolla. [A (floral) character has possible values or states, each of which is called its particular character state or characteristic or trait, the example above meaning that monosymmetry and polysymmetry are just two possible character states or traits of the flower character called symmetry.] In *Rhododendron,* monosymmetric corolla, reflexed stamens, bundled stamens, and reflexed styles do separately give rise to overall monosymmetry of the flower. The study found that each of the four monosymmetry traits was significantly evolutionarily correlated with the presence of pigment patterns on the corolla, as found in a phylogenetic tree of 96 species of *Rhododendron*. It was also noted that these morphological changes are very labile, and that multiple transitions have occurred amongst these floral symmetry traits (Berry *et al.* 2018).

Here, we expanded on that study to combine the above five floral characters, namely, corolla symmetry, flexion of stamens, bundling of stamens, flexion of style, and presence of corolla pigment pattern, into a single five-fold character called a "character-complex" (Cc) for convenience, and analyzed this character complex in the context of

previous information of pollinators, with the objective of better understanding floral morphological aspects of plant-pollinator dynamics. To be precise, the purpose of this study was to address the following questions: i) What meaningful information could be extracted from the representational distribution of the various character state combinations of the five floral characters, i.e., of the character-complex (Cc) states explained above? ii) How are these Cc states distributed on the phylogeny of *Rhododendron*? and iii) Are any of these Cc states or their groups associated with specific pollinators as to be identifiable as pollination syndromes?

Methods

Taxon sampling

To study the distribution of Cc states of the four symmetry attributes together with presence of pigment patterns on corolla in *Rhododendron*, and to understand the relationship between floral Cc states and pollinator types, two datasets were compiled – one, Cc states were coded for 83 species of *Rhododendron* (plus the outgroup species) for mapping on the phylogenetic tree (after Berry *et al.* 2018); two, fresh scoring was done for an additional 25 species for which pollinator information was available in the literature. Coding was done based on flower photographs from published and online sources, as done in Berry *et al.* (2018).

Character-complex states (Cc states): coding and distribution

Five floral characters were combined into a character-complex (Cc) given by an ordered 5-tuple of characters, namely, (corolla symmetry, flexion of stamens, arrangement of stamens, flexion of style, and presence/absence of corolla pigment pattern); and each ordered 5-tuple of the five individual character states (taken in the definite order of the five component characters in the Cc) was called a "Cc state." The five floral characters were coded as follows (after Berry *et al.* 2018):

(i) corolla symmetry: monosymmetric (bilaterally symmetric or zygomorphic) 0, and polysymmetric (radially symmetric or actinomorphic) 1;

(ii) flexion of stamens: reflexed (abaxial flexion) 0, hooked (slight flexion abaxially, but only at the tip) 1, and straight 2;

(iii) arrangement of stamens: bundled 0, loosely bundled 1, and not bundled 2;

(iv) flexion of style: reflexed (abaxial flexion) 0, hooked (slight flexion abaxially, but only at the tip) or straight 1, and deflexed (adaxial flexion) 2;

(v) corolla pigment pattern: absent 0, and present 1.

As explained above, a particular combination of five character states (i.e., an ordered 5-tuple or a 5-sequence of code values), one each for the five characters in the specific order mentioned, was taken to depict a possible Cc state, which was called representable
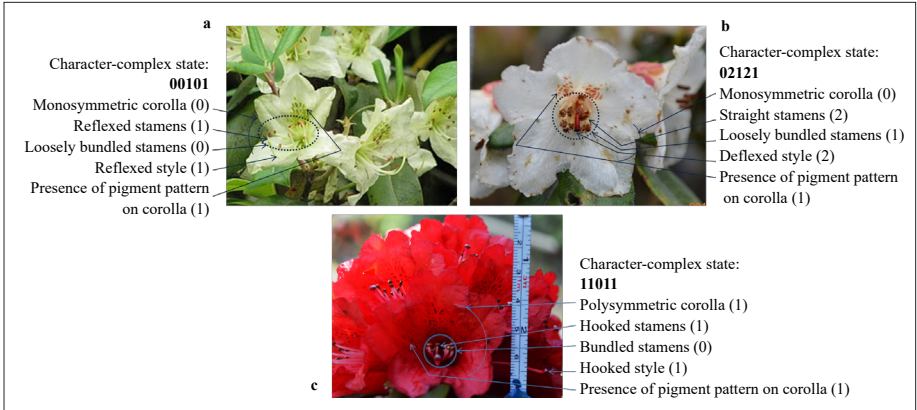
Fig. 1 Illustration of our coding of the floral character-complex (Cc) showing three examples of ordered 5-tuples of character-complex (Cc) states. Three *Rhododendron* species: a. *R. triflorum,* b. *R. pendulum*, c. *R. arboreum* are shown here. The five-component Cc: corolla symmetry, stamen flexion, stamen bundling, style flexion, and pigment pattern on corolla, was coded in a combination, using Berry *et al.* (2018), to yield five component codes in the stated order making each Cc state an ordered 5-tuple of character values.

if found to correspond to, i.e., exhibited through, at least one of the species of our study; e.g., 00101 (Fig. 1). Theoretically, there are 108 possible Cc states, i.e., 2*3*3*3*2, the multiplication result of two states for corolla symmetry, three for stamen flexion, three for arrangement of stamens, three for style flexion, and two for pigment patterns. We coded a total of 109 species for Cc states. *Empetrum nigrum* (crowberry, Ericaceae) was included as the outgroup species.

The maximum likelihood (ML) majority-rule consensus tree (Berry *et al.* 2018) was used to partly show the distribution of Cc states. The 25 additional species were similarly coded to add to the representational distribution picture of the Cc states and to associate it with pollinator information.

Pollinator information and altitude range of the study species were obtained from published work and online sources. This information was related with Cc state information for the different species to determine any pattern between floral Cc traits and pollinator types.

Results and Discussion

Out of the 108 theoretically possible Cc states only 33 Cc states (less than 1/3) were found to occur in the 84 species of *Rhododendron* assessed and with *Empetrum nigrum*. This means only 33 Cc states were found representable. Table 1 lists the Cc states and the *Rhododendron* species that exhibit these combinations. In the character state coding

Table 1. List of character-complex (Cc) states, with numbers and names of *Rhododendron* species representing those Cc states. Cc states and species names in red indicate additional species not present in our phylogenetic analysis in Fig. 2. There is one outgroup species used in the analysis, the crowberry *Empetrum nigrum,* which is a close relative to *Rhododendron* chosen for study. The outgroup species helps in rooting the tree, and in understanding the monophyly of our species.

Cc state	Symbol for Cc state	Number of examined species for Cc state	Representing *Rhododendron* species
00101	Blue circle: ●	18	*R. albrechtii*, *R. canadense*, *R. catawbiense*, *R. dalhousieae*, *R. farrerae*, *R. indicum*, *R. kaempferi*, *R. macrosepalum*, *R. maximum*, *R. mucronulatum*, *R. ovatum*, *R. ponticum*, *R. reticulatum*, *R. rubropilosum*, *R. simsii*, *R. trichanthum*, *R. triflorum*, and *R. vaseyi*
11011	Brown rectangle: ▬	17	*R. arboreum*, *R. barbatum*, *R. beesianum*, *R. delavayi*, *R. elliottii*, *R. floccigerum*, *R. hodgsonii*, *R. hyperythrum*, *R. lacteum*, *R. macabeanum*, *R. maculiferum*, *R. neriiflorum*, *R. pseudochrysanthum*, *R. rex, R. sinogrande*, *R. thomsonii*, and *R. wardii*
00001	Green rectangle: ▬	11	*R. calendulaceum*, *R. camtschaticum*, *R. canescens*, *R. lutescens*, *R. luteum*, *R. macrophyllum*, *R. molle*, *R. nudiflorum*, *R. occidentale*, *R. schlippenbachii*, and *R. stamineum*
12210	Pink rectangle: ▬	6	*R. dielsianum*, *R. lapponicum*, *R. micranthum*, *R. orthocladum*, *R. setosum*, and *R. xanthostephanum*
12110	Pink circle: ●	5	*R. albiflorum*, *R. cinnabarinum*, *R. nipponicum*, *R. radians*, and *R. spinuliferum*

Table 1 continued

Cc state	Symbol for Cc state	Number of examined species for Cc state	Representing *Rhododendron* species
10101	Blue rectangle:	5	*R. adenopodum*, *R. kiusianum*, *R. makinoi*, *R. moulmainense*, and *R. mucronulatum*
11010	Blue spiral:	5	*R. cyanocarpum*, *R. degronianum*, *R. ferrugineum*, *R. pachysanthum*, and *R. wardii*
01111	Green circle:	4	*R. aureum*, *R. brachycarpum*, *R. minus var. chapmanii*, and *R. tashiroi*
11111	Red spiral:	4	*R. clementinae*, *R. konori*, *R. moupinense*, and *R. roxieanum*
10001	Green spiral:	4	*R. excellens*, *R. formosanum*, *R. nuttallii*, and *R. smirnowii*
11210	Brown circle:	4 (3 plus one, outgroup)	*Empetrum nigrum* (outgroup species, *R. hypoleucum*, *R. impeditum*, and *R. tomentosum*
00201	Grey star:	3	*R. hongkongense*, *R. maximum*, and *R. semibarbatum*
02121	Green star:	3	*R. baileyi*, *R. pendulum*, and *R. trichocladum*
10100	Brown star:	3	*R. keiskei*, *R. kiusianum*, and *R. leptothrium*
12120	Blue star:	2	*R. afghanicum*, and *R. virgatum*

Table 1 continued

Cc state	Symbol for Cc state	Number of examined species for Cc state	Representing *Rhododendron* species
12220	Orange star: ⭐	2	*R. campylogynum, and R. genestierianum*
12221	Black star: ★	2	*R. campylogynum*, and *R. sulfureum*
01101	Pink star: ⭐	2	*R. campanulatum*, and *R. mekongense*
00100	Yellow star: ⭐	2	*R. keiskei*, and *R. wadanum*
11211	Red hollow square: ▫	2	*R. oreotrephes*, and *R. ungernii*
00000; 01000; 11110; 01011; 10000; 12011; 12111; 02221; 01001; 02101; 01211; 11221; 12101; 00021; 02010	Black hollow square: ▫	1, for each of the 15 Cc states listed in Column 1	*R. canescens; R. ciliatum; R. cinnabarinum; R. edgeworthii; R. formosanum; R. forrestii; R. konori; R. lepidotum; R. maddenii; R. mekongense; R. siderophyllum; R. sulfureum; R. veitchianum; R. nudiflorum*; and *R. carringtoniae*, respectively for the states mentioned in column 1.
	Question mark: ?	14, the number of species for which we do not have coding, i.e., for which no Cc states could be assigned.	*R. benhallii; R. menziesii; R. anthopogon; R. asperulum; R. championae; R. crassiflolium; R. herzogii; R. kongboense; R. mariesii; R. santapaui; R. sargentianum; R. sororium; R. tsusiophyllum*; and *R. vaccinioides*

within the data from Berry *et al.* (2018) fourteen of the species in that study could not be coded for Cc states due to uncertainty of states for some characters.

Cc states receiving representation are marked for ease of reference with coloured symbols, as defined in Table 1, on a consensus tree from an ML phylogenetic analysis of molecular data from Berry *et al.* (2018). It can be noted from the distribution of Cc states with high abundance that some are well-distributed across the tree while others are confined to particular clades.

Floral Cc state 00001 (symbol: green rectangle), described as monosymmetric corolla, reflexed stamens, bundled stamens, reflexed style, and presence of pigment pattern on corolla, was present in all the major clades of the phylogenetic tree (designated A, B, C in the study) and also in *R. camschaticum*; this state, monosymmetric in *all* components, happens to be the second most abundant state in our analysis (Fig. 2). Cc state 00101 (blue circle), i.e., monosymmetric corolla, reflexed stamen, loosely bundled stamens, reflexed style, and presence of pigment pattern, was also present in clades A, B, and C and was the most abundant state in our study. The third most abundant Cc state 11011 (brown rectangle) was confined to clade B, i.e., subgenus *Hymenanthes*, and can be described as: a polysymmetric corolla with androecium bundling, resulting in effective floral monosymmetry, together with components of slight monosymmetric flexion (i.e., hooked flexion) of stamens and styles, and finally with presence of corolla pigment pattern. The fourth most abundant Cc state 12210 (pink rectangle) was confined to clade A, i.e., subgenus *Rhododendron*. Thus, we can see that some Cc states are widely distributed across the phylogenetic tree, while some others are confined to single clades. This shows that the frequent or favored suites of traits or Cc states are differentially established – either being confined to a single clade, pointing to the possibility of specialized pollinator preference, or occurring across clades and indicating generalized pollinators.

We have information on pollinators that is relatable to some of the representable Cc states by various species of *Rhododendron* (Table 2). There are some noticeable trends of association between the floral Cc states and the types of pollinators visiting those flowers (Table 2).

In the pollinator association study, the most abundant Cc state, namely 00101 (blue circle), i.e., monosymmetric corolla, reflexed stamens, loosely bundled stamens, reflexed style, and presence of pigment pattern was found to be associated with insect pollination. Cc state 11011 (brown rectangle), i.e., polysymmetric corolla, hooked stamens, bundled stamens, hooked/straight style, and presence of pigment pattern, was predominantly found to be associated with bird pollination, while its single-character-state variant, 11010 (blue spiral), different in having an absence of pigment pattern,

Fig. 2. (caption on next page)
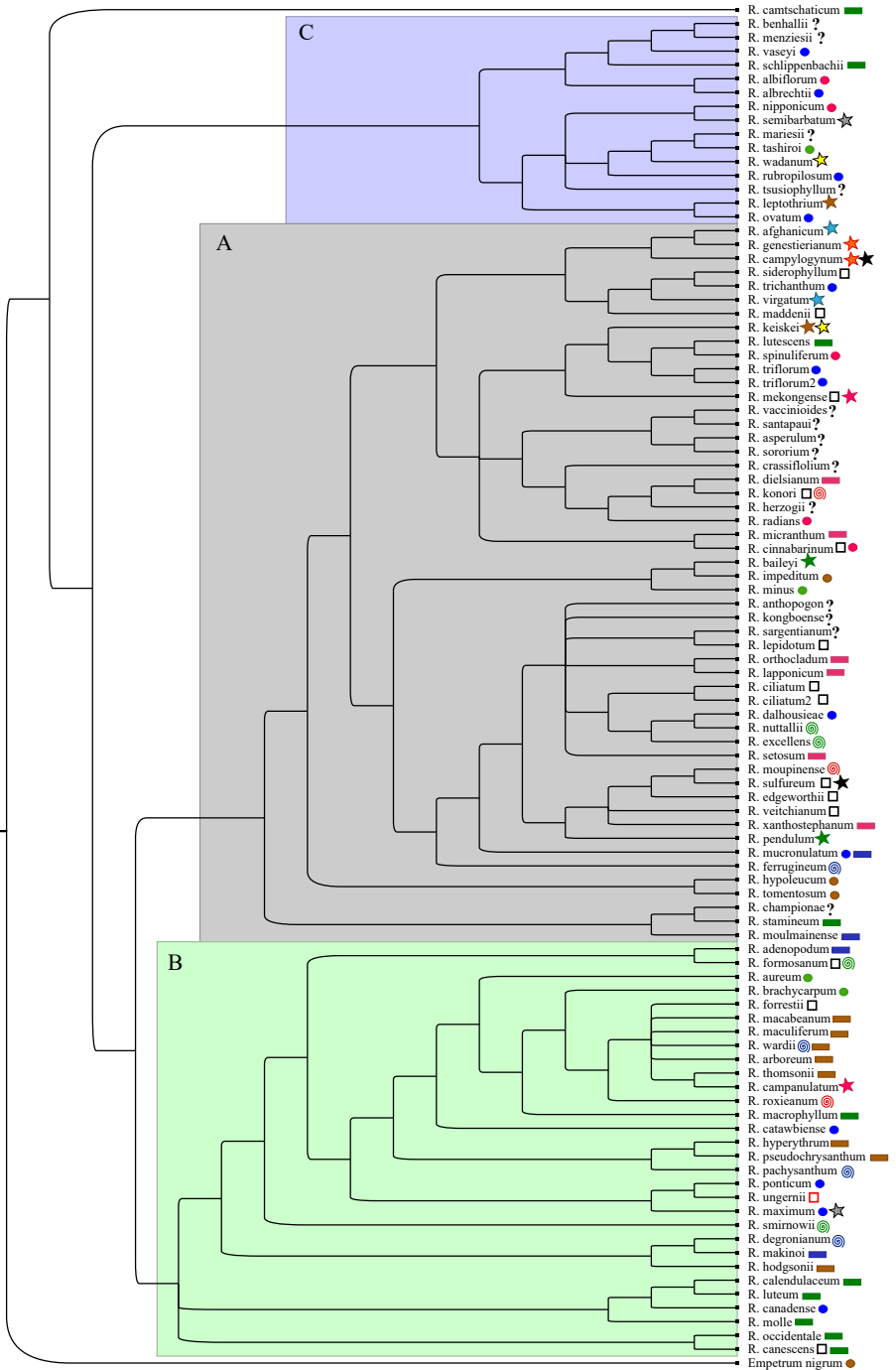
Fig. 2. Distribution of character-complex (Cc) states on a phylogenetic tree of *Rhododendron* species. The three major clades A, B and C are coloured grey, green, and blue. Clades A and B comprise two major subgenera *Rhododendron* and *Hymenanthes* respectively. The different character-complex states that receive representation on the tree have been indicated through various coloured symbols of filled-in circles, rectangles, spirals, stars and hollow squares. An occurrence of these coloured symbols beside a taxon name shows the Cc state of the species. Eleven species names, each accompanied with two Cc state symbols, indicate presence of polymorphism in these species. The set of representing species includes the outgroup species. Species on the tree (14 in number) for which Cc state could not be ascertained are shown accompanied with the special symbol '?'. Please refer to Table 1 to find a list of all the Cc states that have found occurrence, i.e., representation, in the *Rhododendron* species studied. The coloured symbols reveal how the Cc states are differently represented on the tree: some CC states find representation in all major clades, some more in only one major clade, while many more do not find any representation at all. Out of the 108 theoretically possible Cc states, only 33 Cc states (less than 1/3rd) can be seen to be exhibited in the 82 coded *Rhododendron* species studied here.

was found to be associated with both bird and insect pollination. Another example, from the literature, of a Cc state that shows possible association with bird pollination is 11111 (red spiral), which in totality is slightly less monosymmetric (stamens are loosely bundled) than the predominantly bird-pollinated character-complex state 11011 (brown rectangle) in which stamens are bundled. This suggests that pollinator associations in some cases are spread over multiple Cc states, like with 11111 and 11011, suggesting interesting groups or suites of traits for pollination syndrome study, and this might also mean that some minor trait transitions do not affect pollinator associations. One can also note that unlike the Cc state 11011, which is confined to *Hymenanthes*, the Cc state 11111 is found to be more widely distributed on the phylogenetic tree, occurring in two large clades formed of subgenera *Hymenanthes* and *Rhododendron*.

For the Cc state 00001 (green rectangle), which was found common to clades A, B and C, and was associated in general with insect pollination, it can be remarked, as earlier, that it is characterized by strongest evidence of monosymmetry. Two representing species were found to be associated with butterfly pollination; of these, *R. nudiflorum* shows polymorphism in the flexion of style, with both reflexed and deflexed styles present. Here, the style is initially deflexed during the male stage, and in the female stage, it flexes abaxially and becomes reflexed. This type of stylar movement is thought to help ensure cross-pollination and to minimize self-pollination (Leppik 1974).

Table 2 lists a representable character-complex (Cc) state 02010 (red double asterisk) not represented on our *Rhododendron* species phylogenetic tree. This Cc state gives at least one example that supports the assertion that there do exist more representable Cc states over the full genus *Rhododendron* than are actually represented on our phylogenetic tree. When one notes that our phylogenetic tree sampling was only of about 10-17% of the total species diversity in *Rhododendron* (depending on 1000 or

Table 2. Character-complex (Cc) states examined, *Rhododendron* species representing them, their associated pollinator types, insect or bird (highlighted through colour), including pollinator species names and plant species altitudinal range. $^{\Omega}$ indicates 25 new *Rhododendron* species for which Cc state scoring is provided, i.e., in addition to the species from Berry *et al.* (2018). These species were included to utilize available pollinator information for them, and to analyze resultant trait (Cc state) - pollinator group associations (shown through use of the symbol '↔'). ¥ stands for www.hirsutum.info, our source for altitudinal range data. $^{£}$ indicates new representable Cc state (namely, 02010, here accompanied with a new symbol 'red double asterisk' i.e., **) found in addition to the Cc states observed in the 96 species from Berry *et al.* (2018). This underlines that the addition of 25 *Rhododendron* species does add to the total number of represented Cc states in our study.

Floral Cc state (with attributed symbol)	Representitive *Rhododendron* Species, known associated pollinators, and species altitudinal ranges	Reference
11010 ◉	$^{\Omega}$*R. cyanocarpum* ↔ Insect pollinators [Bumble bees: *Bombus festivus* and *B. richardsiellus*]	Ma *et al.* 2015
	R. cyanocarpum ↔ Bird pollinators [*Phylloscopus affinis*, *Pycnonotus jocosus monticola*]. (3400-4000 m)	Huang *et al.* 2017
	R. ferrugineum ↔ Insect pollinators [Honey bees and Bumble bees]. (1600-2200 m)	Escaravage and Wagner 2004; http://www.iucnredlist.org
	R. wardii ↔ Insect pollinator(s) (Bird pollination suggested!). (2700-4300 m)	Huang *et al.* 2017; ¥
11011 ▬	$^{\Omega}$*R. barbatum* ↔ Bird pollinator [*Zosterops japonicas*]. (2400-3700 m)	Huang 2011; ¥
	$^{\Omega}$*R. beesianum* ↔ Bird pollinators [*Aethopyga gouldiae*, *Phylloscopus affinis*, *Zosterops japonicas*]. (3000-4400 m)	Huang *et al.* 2017; ¥
	$^{\Omega}$*R. delavayi* ↔ Bird pollinators [*Heterophasia melanoleuca*, *Pycnonotus jocosus monticola*]. (2200-2500 m)	Huang *et al.* 2017
	$^{\Omega}$*R. elliottii* ↔ Bird pollinator [*Heterophasia pulchella*] and Insect pollinators [Carpenter bees, *Xylocopa* spp.]. (2400-2700 m)	Jing *et al.* 2015; ¥

Table 2 continued

11011 cont.	$^{\Omega}$ *R. floccigerum* ↔ Insect pollinator [Bombus spp.] and Bird pollinators [*Aethopyga gouldiae*, *Garrulax affinis*, *Heterophasia melanoleuca*, and *Yuhina diademata*]. (2700-4000 m)	Georgian *et al.* 2015; ¥
	$^{\Omega}$ *R. lacteum* ↔ Bird pollinators [*Aethopyga ignicauda*, *Pycnonotus jocosus monticola*]. (3000-4000 m)	Huang *et al.* 2017; ¥
	R. macabeanum ↔ Bird pollinators [*Yuhina* spp.]. (2400-3000 m)	Jing *et al.* 2015; ¥
	$^{\Omega}$ *R. neriiflorum* ↔ Bird pollinators [*A. ignicauda*, *P. jocosus monticola*]. (2550-3600 m)	Huang *et al.* 2017
	$^{\Omega}$ *R. rex* ↔ Bird pollinators [*A. ignicauda*, *P. affinis*]. (3000-4300 m)	Huang *et al.* 2017; ¥
	$^{\Omega}$ *R. sinogrande* ↔ Bird pollinators [*A. ignicauda*, *Yuhina occipitalis*]. (2100-3400 m)	Huang *et al.* 2017; ¥
	R. wardii ↔ Insect pollinator(s) (Bird pollination suggested!). (2700-4300 m)	Huang *et al.* 2017; ¥
00101 ●	$^{\Omega}$ *R. farrerae* ↔ Insect pollinators [*Bombus eximius* and *Xylocopa* spp.]	Ng and Corlett 2000
	$^{\Omega}$ *R. indicum* ↔ Insect pollinators [bees and butterflies]	Tagane *et al.* 2008
	$^{\Omega}$ *R. kaempferi* ↔ Insect pollinators [Butterflies and Honey bees]. (0-1200 m)	Yokogawa and Hotta 1995; ¥
	$^{\Omega}$ *R. macrosepalum* ↔ Insect pollinators [Bumblebee: *Bombus ardens ardens*]	Sugiura 2012
	R. ponticum ↔ Insect pollinators [Bumblebees and Carpenter bees]. (0-1800 m)	Stout *et al.* 2006; ¥
	$^{\Omega}$ *R. reticulatum* ↔ Insect pollinators [Beefly: *Bombylius major*]. (0-1800 m)	Sugiura 2012; ¥
	$^{\Omega}$ *R. simsii* ↔ Insect pollinator(s). (300-2400 m)	Huang *et al.* 2017; Ng and Corlett 2000; ¥

Table 2 continued

00201	☆	$^{\Omega}$ *R. hongkongense* ↔ Insect pollinators [*Bombus eximius* and *Xylocopa* spp.]	Ng and Corlett 2000
		R. semibarbatum ↔ Insect pollinators [Bumblebees: *Bombus ardens* and *Bombus honshuensis*]. (200-1000 m)	Ono *et al.* 2008
01111	●	*R. aureum* ↔ Insect pollinators [Bumblebee queens and flies]. (1500-2700 m)	Hirao *et al.* 2006; ¥
00001	▬	*R. calendulaceum* ↔ Insect pollinators [Butterflies: *Papilio glaucus* and *Speyeria cybele*]. (200-1000 m)	Epps *et al.* 2015; ¥
		$^{\Omega}$ *R. nudiflorum* ↔ Insect pollinators [Syrphid fly: *Doros aequalis*, and Butterflies]	Leppik 1974
11111	◎	$^{\Omega}$ *R. clementinae* ↔ Bird pollinators [*Aethopyga gouldiae*, *Phylloscopus affinis*]. (3300-4200 m)	Huang *et al.* 2017
02121	★	$^{\Omega}$ *R. trichocladum* ↔ Insect pollinator(s). (3200–3600 m)	Huang *et al.* 2017
12120	★	*R. virgatum* ↔ Insect pollinator(s). (2200–2800 m)	Huang *et al.* 2017
10100	★	$^{\Omega}$ *R. kiusianum* ↔ Insect pollinator(s) [Bees]. (1200-1700 m)	Yokogawa and Hotta 1995; ¥
10101	▬	$^{\Omega}$ *R. kiusianum* ↔ Insect pollinator(s) [Bees]. (1200-1700 m)	Yokogawa and Hotta 1995; ¥
		R. moulmainense ↔ Insect pollinator(s)	Ng and Corlett 2000
00021	☐	$^{\Omega}$ *R. nudiflorum* ↔ Insect pollinators [Syrphid fly: *Doros aequalis*, and Butterflies]	Leppik 1974
11211	☐	$^{\Omega}$ *R. oreotrephes* ↔ Bird pollinators [*Aethopyga gouldiae*, *Phylloscopus affinis*]. (2700-4300 m)	Huang *et al.* 2017; ¥
$^{£}$**02010** ******		$^{\Omega}$ *R. carringtoniae* ↔ Insect pollinators [Butterflies]	Leppik 1974

600 as the total count of the species in the genus), this seems unsurprising, though corroborated by a simple example.

As suggested by a study of 15 *Rhododendron* species (Huang *et al.* 2017), other published works, and additional altitudinal data from online sources, it can be inferred that in general, bird pollination is found in those species of *Rhododendron* that are at higher altitudes when compared to insect pollinated rhododendrons (Table 2). Our own field observations in the Sikkim Himalayas also indicated that flowers with the Cc state 11011 (brown rectangle) are found to be primarily visited by bird pollinated species unique to *Hymenanthes*, having an altitudinal range of 3000 to 4500 m, and are inferred to be predominantly pollinated by birds in Table 2. No pollinator information is available for flowers with the Cc state 12210 (pink rectangle), e.g., *R. setosum*, which occurs at altitudes of up to 5000 m.

Conclusion

We have demonstrated a novel method structure of observing morphological characters and their traits or character states in *Rhododendron*, by combining characters into a character-complex (Cc) that has many Cc states or suite of traits. We find for the first time signals of pollination syndromes in *Rhododendron*.

Subtle changes in form and structure of *Rhododendron* flowers, like any group of angiosperms displaying such diversity, can be variously explored and analyzed for study of the evolution of plant-pollinator interaction. Questions related especially to our work and still to be addressed include "What makes these character-complex states so labile? Out of so many possible combinations of Cc states, why should there be only a few that seem to be actually represented in the genus *Rhododendron*? Presumably, these questions relate to the origin and development of morpho-states, and their feasibility and non-feasibility in character morphospace in the context of the evolution of groups and subgroups of the genus. Although there is obvious and expected overlap in the pollinator types visiting flowers with different, close and not-so-close, Cc states, there are noticeable morpho-signals in these morpho-states that suggest predominant pollinator associations. This paper admittedly does not delve deeply into this question. As for the study of pollination syndromes, each Cc state or a suitable group of such Cc states could be a candidate for the study only if a one-to-one pollinator and Cc state association emerges. It is a limitation of this work that beyond general hints of correspondence no tight pollinator association could be underlined that could lead to intensive pollination syndrome study. Further ecological studies would be needed to extract more information on pollinator association.

Finally, it seems reasonable to state the truism that there is much information inside the morphology of a flower that is intuitively and more straightforwardly observational,

even in the presence of facilitating, modern, and sophisticated tools and techniques. Moreover, a balanced support of intuition and technique can help address more challenging questions in a highly diverse plant group like *Rhododendron*, making this type of exploration even more rewarding.

Acknowledgement

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A Look at Hybrid Zones in *Rhododendron*, with Particular Reference to F1-Dominated Hybrid Zones (F1DZs)

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Introduction

Rhododendrons are famous for their ability to hybridize, and were among the first garden plants subjected to intensive between-species breeding programs during the 19th Century. Where hybridization occurs in the wild, it can lead to large populations of what are termed hybrid derivatives, i.e., individuals that have more than one species in their immediate ancestry. A patch of habitat containing large numbers of hybrid derivatives is termed a "hybrid zone," and where those hybrids display great variation in form and genetic composition, as a result of hybridization having proceeded beyond the first generation, the hybrid population is also termed a "hybrid swarm." Where hybrids exist in any quantity, a hybrid swarm is the most usual configuration for the hybrid population, for reasons explained below. Many hybrid zones in *Rhododendron*, however, exhibit highly unusual population structures, so this review will focus, in particular, upon a form of hybrid zone that is relatively common in *Rhododendron* but extremely rare outside it: the F_1-dominated hybrid zone (F_1DZ).

Natural Hybrid Formation in *Rhododendron*

Only relatively closely related species are capable of forming hybrids, and even this ability varies greatly across genera and families (Ellstrand *et al.* 1996, Whitney *et al.* 2010). *Rhododendron* species are among the least faithful of all, and as a rule of thumb *Rhododendron* species tend to be able to form fertile hybrids with most or all members of their own subgenus. For example, *R. ponticum* is separated from most other species

of its subgenus, *Hymenanthes*, by around ten million years of evolution (Milne 2004) but forms fertile hybrids with all three of the *Hymenanthes* species that it grows with in the wild (*caucasicum*, *smirnowii* and *ungernii*; Milne *et al*. 1999), and many more in cultivation (Milne and Abbott 2000, van Ree 2015, Milne 2017). Hybrids between subgenera can occur but are rare, often sterile and in many cases only exist due to deliberate human efforts, e.g., subgenus *Hymenanthes* × *Pentanthera* (Milne 2017), subgenus *Tsutsusi* × former genus *Menziesia* (Kita *et al*. 2005), and *Tsutsusi* × subgenus *Rhododendron* section *Schistanthe* (Eeckhaut *et al*. 2013). Evidence of past hybridization and gene flow between species (introgression) may often be detected with molecular markers (Huang *et al*. 2011; and see Table 1 for more *Rhododendron* examples), and ancient hybridization is indicated for subgenus *Hymenanthes* by strong discordance between relationships indicated by cpDNA and those from morphology (Milne *et al*. 2010) or nuclear RPB2 markers (Goetsch *et al*. 2005). Several species appear to have been derived via hybrid speciation; these include the vireyas *R. sarcodes, R. arenicola, R. pseudobuxifolium* and *R. stapfianum* (Goetsch *et al*. 2011), and probably *R. balangense* in *Hymenanthes* (Milne, 2017). This phenomenon should not be confused with early generation hybrids being erroneously classified as species, which has happened many times in *Rhododendron*.

The question, therefore, is not so much why *Rhododendron* hybrids form, but how species in general avoid merging, or being swamped by their own hybrids in the wild. A systematic survey of breeding barriers between *R. cyanocarpum* and *R. delavayi* revealed considerable overlap in flowering time and pollinating species, the latter despite *R. delavayi* being more adapted for bird pollination and *R. cyanocarpum* more adapted for bees (Ma *et al*. 2016). Hybrid seed had lower viability than non-hybrid seed, but enough would germinate to make this a weak barrier as well (Ma *et al*. 2016). More significant, perhaps, was that conspecific pollen formed pollen tubes faster than heterospecific pollen (a well-known mechanism, e.g., see Arnold 2000), meaning no hybrids would be formed if the two kinds of pollen were deposited at the same time (Ma *et al*. 2016). However, many flowers went unpollinated, meaning there would be opportunities for heterospecific pollen to be the only pollen that reached a flower, allowing hybrids to be sired (Ma *et al*. 2016). The most significant barrier was pollinator constancy: individual insects tended to be faithful to a single species and were not observed switching between them (Ma *et al*. 2016). Hence, some *Rhododendron* in the wild may rely on insect behavior, and the fact that they look different to insect visitors, in order to minimize pollen transfer between species, and hence hybridization.

We cannot be certain how universal this effect is, but it is likely to be highly significant for any species pair where the parents have very different looking flowers (e.g., *R. spiciferum* and *R. spinuliferum*), where they tend to be spatially separated, or both (e.g.

Table 1: Cases of hybridization in *Rhododendron* where information exists on hybrid zone structure and/or introgression.

Parent Species	Habitat for parents	Habitat for hybrids	Hybrid zone structure	Backcrossing	Introgression	Evidence	Reference
flammeum X *canescens*	Well-drained bluffs; floodplains or low wet forests.	Mesic forest on slope overlooking artificial lake.	Not determined	Class not determined	Into *flammeum*	cpDNA	Kron *et al.* 1993
smirnowii X *ungernii*	Usually on rocks 1500 - 2300 m; mostly in woodland, 1200 - 1850 m.	Natural to mildly degraded vegetation	Not determined; scattered individuals	Class not determined	Into *ungernii*	rDNA RFLP marker, flower colour	Milne *et al.* 1999
ponticum X *ungernii*	Woodland below 1800 m; mostly in woodland 1200 - 1850 m.	Natural to mildly degraded vegetation	Not determined; scattered individuals	Class not determined	Both ways	cpDNA in *ungernii*; rDNA RFLP marker and calyx lobes in *ponticum*	Milne *et al.* 1999
ponticum X *caucasicum* (at Çamlıhemşin, Turkey)	Woodland below 1800 m; alpine slopes above 1700 m	Natural, bottom of steep gulley with late-lying snow, c. 1700 m	Morphological variety suggests a segregating hybrid swarm	Both directions (probable)	To *ponticum* (very rare)	rDNA RFLP marker	Milne *et al.* 1999
ponticum X *caucasicum* (at Tiryal Dag, Turkey)	Woodland below 1800 m; alpine slopes above 1700 m	Slopes between parents, 1900 - 2200 m	All F₁s (F₁DZ)	None detected here.	None detected here	Nuclear RAPD markers	Milne *et al.* 2003
eriocarpum X *indicum*	Coastal; riversides.	Seaside area.	Not determined, but variation suggests a segregating hybrid swarm	Both directions (probable)	To *eriocarpum* (implied)	No direct evidence given; PCo on AFLPs strongly implies backcrossing.	Tagane *et al.* 2008
spiciferum X *spinuliferum*	Unclear.	Often disturbed	Hybrid swarms with ample backcrossing	Both directions	Likely but not confirmed	Microsatellites and cpDNA.	Yan *et al.* 2017.
kaempferi X *kiusanum*	440 – 600 m; 1030 – 1650 m at hybrid zone on Mt. Kirishima	Slopes at intermediate altitudes. Hybrid zone known to be >200 years old.	Morphological variety suggests a segregating hybrid swarm	Class not determined	Ancient, into *kiusanum*	cpDNA	Kobayashi *et al.* 2000; 2007.
ferrugineum X *hirsutum*	Acid soils; basic soil.	Intermediate pH.	Some sites, 57% F₁s and otherwise mainly backcrosses to *hirsutum*; others more mixed	Mostly or only towards *hirsutum*	Only towards *hirsutum*	RAPD markers, cpDNA.	Milne and Abbott, 2008; Bruni *et al*, 2016.
Phaeochrysum X *aganniphum* (Baima Shan 'Hyb-1')	Both mountain slopes, but *aganniphum* higher up	Mountain slopes, mixed with both parents.	Hybrid swarm with many backcrosses.	Most or all towards *aganniphum*	Probable in both directions, at least in the past.	AFLP markers.	Marczewski *et al.* 2015
Phaeochrysum X *aganniphum* (Baima Shan 'Hyb-2')	Both mountain slopes, but *aganniphum* higher up	Mountain slopes, away from parents.	Either all F₁s, or a self-contained, possibly stabilizing hybrid swarm.	None detected here.	Unlikely at this site.	AFLP markers.	Marczewski *et al.* 2015
Periclymenoides X *atlanticum*	Upland or sandy woods, bluffs, stream banks; sandy pinelands & wet places	Not specified	Not determined, probably hybrid swarm.	Both ways	Probably both ways, not confirmed	Morphometric analysis and flavonoids.	King, 2000.
ripense X *macrosepalum*	Riverside; forest.	Not specified	Not specified	Class not determined	Into *ripense* (one sample)	CpDNA and nrDNA.	Yokoyama *et al.*, 2012.
delavayi X *irroratum*	Both form woods on slopes in the area.	Wide area, mildly disturbed	Probable hybrid swarm	Both ways	Not examined	Detailed morphology	Marczewski *et al.* 2016

R. ponticum and *R. caucasicum*); however both of these examples still produce hybrids in large numbers (Yan *et al.* 2017; Milne *et al.* 2003). Moreover, it is unclear how strongly this barrier would apply where the parent species have similar looking flowers (at least to human eyes), and/or sometimes occur intermixed, as in *R. ferrugineum* and *R. hirsutum* (Milne and Abbott 2008), and *R. aganniphum* and *R. phaeochrysum* (Marczewski *et al.* 2015). This barrier was observed to break down easily between *R. eriocarpum* and *R. indicum*, for example, leading to frequent hybrid zones where they intermix (Tagane *et al.* 2008).

In other species pairs, temporal isolation might be more significant. For example, *R. ferrugineum* flowers around a month earlier than *R. hirsutum*, and they barely overlap (Milne and Abbott 2008). The last flowers of *ferrugineum* and the first of *hirsutum* might, however, be outnumbered by pollen of the other species, weakening the effect of the pollen tube growth speed barrier, and hence promoting hybrid formation during the brief period of overlap. Both species are protandrous (i.e., stamens mature before female parts), and hence the last stigmas of *R. ferrugineum* should therefore receive a lot of *R. hirsutum* pollen, whereas when the first stigmas of *R. hirsutum* become receptive, there should already be conspecific pollen about. This should lead to *ferrugineum* being the mother of most hybrids, because cpDNA is maternally inherited. However, hybrids normally have *R. hirsutum* cpDNA (= chloroplast DNA; Bruni *et al.* 2016), with *R. hirsutum* normally the hybrid mother, unless inheritance is paternal, as reported in crosses involving *Menziesia* (now included within *Rhododendron*) *multiflora* (Kita *et al.* 2005).

Floral morphology may contribute to isolation in some cases, e.g., different style lengths in section *Schistanthe* (previously *Vireya*; Williams & Rouse 1988), although floral adaptation to different pollinator guilds did not seem to have a big effect on hybridization between *R. delavayi and* R. *cyanocarpum* (Ma *et al.* 2016). Likewise, dramatically different flower structure between *R. spiciferum* and *R. spinuliferum* has not prevented the formation of at least 15 populations of hybrids between them (Yan *et al.* 2017)

Loss of hybrid fitness is a well-known mechanism for keeping species separate (Rieseberg and Carney 1998), but *Rhododendron* hybrids are often both fertile and vigorous. Where both species are specialized for particular pollinator types or habitats, hybrids may find neither suitable habitat nor pollinator, as Danet (2012) suggested for vireyas. Habitat transitions, however, often provide ideal habitats for hybrids (see below), and if certain pollinators are shared between parents, then these are likely to visit the hybrids as well (Tagane *et al.* 2008; Ma *et al.* 2014, 2016).

In contrast to these natural species barriers, human disturbance promotes hybridization

in numerous ways: expanding species ranges, altering habitats, introducing or removing pollinators, increasing wildfires, altering climate and hence phenology, or changing the ratio of parent species (Anderson 1948, 1949; Semple and Semple 1977; Rieseberg and Carney 1998; Bleeker and Hurka 2001; Lamont *et al.* 2003; Guo 2014; Ortego *et al.* 2017; Grabenstein and Taylor 2018). Moreover, altered and especially disturbed habitats may favor hybrids, and especially post-F_1 hybrid derivatives, over parental species (Abbott 1992, Arnold 1997; Rieseberg and Carney 1998). Disturbance has probably promoted hybridization in *Rhododendron spiciferum* × *R. spinuliferum* (Li-Jun Yan, pers. comm.) and some vireyas (Danet 2012) but clear-cut examples of this are quite rare in *Rhododendron* overall. This may be because the signature of disturbance becomes harder to detect with time, making it harder to link with hybridization in relatively long-lived groups, like *Rhododendron*. Humans have been disturbing habitats for millenia, especially in densely populated places like China, and the extent to which this has promoted natural *Rhododendron* hybridization has barely been addressed.

Wherever hybridization proceeds beyond the first generation, the potential exists for introgression—gene flow from one species into another. Numerous examples of this are known in *Rhododendron* (Table 1), including the unusual example of *Rhododendron ponticum* in the British Isles, which underwent introgression from multiple species in cultivation, before escaping into the wild (Milne and Abbott 2000). Furthermore, some pairs or groups of species might continuously exchange genetic material, while remaining distinct through ongoing selection on those parts of the genome that code for morphological and ecological differences (Wu 2001). This might apply between *Rhododendron ferrugineum* and *R. hirsutum* (Milne and Abbott 2008, Bruni *et al.* 2016), and strong selection upon particular genetic markers has been detected in hybrid populations of *R. aganniphum* × *R. phaeochrysum* (Marczewski *et al.* 2015). It is highly plausible that ongoing interspecific gene flow might be the norm for many *Rhododendron* species pairs, but this process makes the genetic material of the species concerned increasingly hard to distinguish, and is therefore hard to detect.

In *Rhododendron* more than any other genus, there exists one other extraordinary mechanism that can limit the gene flow between hybridizing species, and that is the exclusion of later generation hybrids by populations of extremely fit F_1s. This is the only mechanism that can explain the existence of F_1-dominated hybrid zones (Milne *et al.* 2003), and these will now be considered in detail.

F_1-Dominated Hybrid Zones (F_1DZs).

F_1DZs are hybrid zones in which all or most hybrids are F_1s, despite those F_1s being highly fertile (Milne *et al.* 2003). They tend to form on undisturbed, transitional habitats between those of the parent species, and typically F_1s will occur in large

numbers, occupying most or all suitable sites. This type of hybrid zone is extremely rare, with only up to ten documented cases across all land plants (Table 2), but it is disproportionately common in *Rhododendron*.

Hybridization always begins with the formation of an F_1 hybrid. Such events are relatively rare because all species have mechanisms that favor crossing with their own kind, as discussed above. Once an F_1 is formed, however, then any and all seed it produces will be hybrid derivatives—either F_2 or backcrosses. The same applies to all its descendants, and hence if the F_1 is fertile, then later generation derivatives (here collectively termed post-F_1s for simplicity) can quickly be produced in large numbers (Rieseberg and Carney 1998). This leads to a "hybrid swarm" of post-F_1s, and is the reason why such swarms are a common result of producing fit F_1 hybrids that can reproduce. Among post-F_1s, and especially F_2s, every individual inherits a unique combination of alleles from the two parent species, leading to massive variation in both form and ecology between hybrid individuals, a phenomenon termed segregation (Anderson, 1948). This can be seen very dramatically in the Kurume Azaleas, a set of cultivars believed to be derived directly from a hybrid swarm between *R. kiusanum* and *R. kaempferi* (Kobayashi *et al.* 2000, Milne, 2017). Each named cultivar is a particular post-F_1 clone, and there is extraordinary variation between them regarding flower colour, size and shape (Fig 1). Therefore naturally formed hybrid swarms tend to exhibit great between-individual variation. There is also often much backcrossing towards one or both parents, further increasing variability (Arnold 1997, Rieseberg and Carney 1998), including among *Rhododendron* (Table 1).

For an F_1DZ to form, there must be repeated formation of F_1s in spite of species barriers, but little or no recruitment of post-F_1s. In theory, a simple mechanism for this is F_1 sterility, as seen for example in *Begonia* (Twyford *et al.* 2015), but often such sterility is not total, allowing occasional post-F_1s to form, from which a hybrid swarm might yet be produced. F_1DZs however contain large numbers of F_1s, and F_1 fertility has been confirmed in most cases of F_1DZs in *Rhododendron,* as well as in other taxa (Table 2). A second possible reason a hybrid zone might have only F_1s is that it is only one generation old, but cases exists where contact is recent but F_2s have already been formed, e.g., with *Salix* hybrids where glaciers have recently retreated (Gramlich and Hörandl 2016), suggesting that the time window for F_1-only populations to exist by this mechanism may be quite short. Moreover, all documented F_1DZs exist on undisturbed habitats, with parent species and sometimes also hybrids (e.g., Jiang *et al.* 2016) appearing to be long-established at the site. It is possible that hybridization could have begun between long-established populations only recently, through a very rare F_1 formation event, but if so, there is no explanation for how large numbers of F_1s could be formed simultaneously and come to occupy all parts of a narrow band

Table 2: Cases of F$_1$-dominated hybrid zones (F$_1$DZs) or possible F$_1$DZs in *Rhododendron* and other genera. Number given is number of F1s/number of hybrid classes detected in total. Where multiple sites examined, one is given after the other.

Hybrid and parents	Major habitat separation factor for parents	Habitat of F$_1$DZ	Location	Proportion of F$_1$s among hybrids at site(s)	Do non-F$_1$-dominated hybrid zones occur for these species?	F$_1$ fertility confirmed?	How many F$_1$ genets examined?[1]	Caveats	Reference
Rhododendron × sochadzeae (= ponticum X caucasicum)	Altitude (very little overlap)	Mountain slopes; F$_1$DZs observed between 1900 and 2200 m	Seaward side of mountains along S Coast of Black Sea	100%	Yes, in steep river valleys at lower altitude.	Yes, experimentally	12/12	Small sample (12 genets) examined.	Milne *et al.* 2003
Rhododendron × intermedium (= ferrugineum X hirsutum)	Soil pH	Sites with mixed soil and varying pH	European Alps (F$_1$DZs examined were south of Innsbruch).	57% (possibly more).	Yes, in the Italian Alps, perhaps promoted by ski-related disturbance	Yes, experimentally	17/21, 17/30	Full dominance by F$_1$s has not been observed.	Milne and Abbott, 2008; Bruni *et al.* 2016.
Rhododendron × agastum (= irroratum X delavayi)	Altitude, but much overlap	Forested hillside in well preserved vegetation.	Hua Dian Ba near Dali, Yunnan, western China	100%, 94%	Huge hybrid population at Baili is probably a hybrid swarm	Yes, experimentally	14/14, 19/21	Baili data based on morphology only	Zha *et al.* 2010; Marczewski *et al.* 2016.
Rhododendron phaeochrysum X aganniphum	Altitude, but much overlap.	Mountain slopes	Baima Shan mountain, Yunnan	Close to 100% but see caveats	Yes – another site on the same mountain exhibits far greater variation among hybrids.	Implied by backcrossing and introgression	35/35 (class not confirmed)	Could also be a population of late generation hybrids in process of homoploid hybrid speciation	Marczewski *et al.* 2015
Encelia × laciniata (= ventorum X palmeri)	Dune stability and moisture content	Stable leeward dune slopes	Baja California, USA.	Probably 100%	Yes, on disturbed sites nearby	Yes, experimentally	Not determined	F$_1$s identified by lack of morphological segregation, and offspring traits, only.	Kyhos *et al.* 1981.
Viola bissett X rossii	Slope aspect	Ridge tops, with parents on slopes either side.	Mount Ougi, Central Japan	Between 81% and 95%	None are known	Not tested as yet.	Not determined	Unclear whether selection vs post-F$_1$s is habitat-mediated	Nagano *et al.* 2015
Populus × jrtyschensis (= nigra X laurifolia)	Wetness and soil nitrogen.	Floodplains, wth less soil nitrogen than parental habitats	Xinjiang, Western China.	84% or more.	None mentioned.	Seeds observed but viability not determined. Backcrosses prove some viability	187/215 (across 14 sites)	F$_1$ habitat extreme: wetter, less nitrogen than either parent's.	Jiang *et al.* 2016.
Phyllodoce caerulea X aleuticum	Altitude	Snowmelt zones on mountains	Taisetsu Mountains, Hokkaido, northern Japan	100% in most sites	None known, but a minority of backcrosses ate some sites.	Yes, experimentally	18/24, 48/57	Authors suspect selection vs post-F$_1$s not habitat-mediated	Kameyama *et al.* 2008; 2011.
Populus canescens (= alba X tremula)	Altitude (*alba* by rivers, *tremula* in hills above)	Riverine lowland forest (often naturally disturbed) mixed with *P. alba* (Europe); along river tributaries (China)	Ticino, Danube Tisca & Irtyish rivers in Italy, Austria, Hungary and China respectively.	85%, 74%, 56% (Europe); 79% (China).	None this author is aware of.	Backcrosses both ways shown to be viable.	73/86, 25/34, 9/16, (Europe) 108/~137 (China)	Selection vs post-F$_1$s may be intrinsic. Hybrid zones are naturally disturbed.	Christe *et al.* 2016 (Europe); Zeng *et al.* 2016 (China).
Salvia × sylvestris (= pratensis X nemorosum)	Soil drainage: moist earth with humus, dry, slightly elevated ground.	Sides of miniature ridges in undulating meadows.	SE Austria, near Hungarian border.	Unknown, appeared to be high	Unknown.	Yes, experimentally	Not recorded	No hard evidence, based on detailed descriptions of sites now presumed destroyed	Kerner, 1895.

of suitable habitat. Clonal spread does contribute to F$_1$ dominance within *Phyllodoce* F$_1$DZs (Kameyama *et al.* 2008) and to a much lesser extent in *Rhododendron* (Milne *et al.* 2003, Milne and Abbott, 2008) and *Populus × jrtyschensis* (Jiang *et al.* 2016). Outside of F$_1$DZs, this process has allowed *Typha × glauca* F$_1$s to become invasive in North America (Zapfe and Freeland 2015, Pieper *et al.* 2018). F$_1$DZs, however, are consistently shown to contain multiple F$_1$ genets when examined with molecular markers (Table 2), so clonal spread alone cannot account for their existence.

To establish a large population of fertile F$_1$s where they greatly outnumber subsequent generations, it therefore seems necessary that F$_1$s would outperform all other genealogical classes (including parents) within the hybrid zone. Certainly they must have habitat-
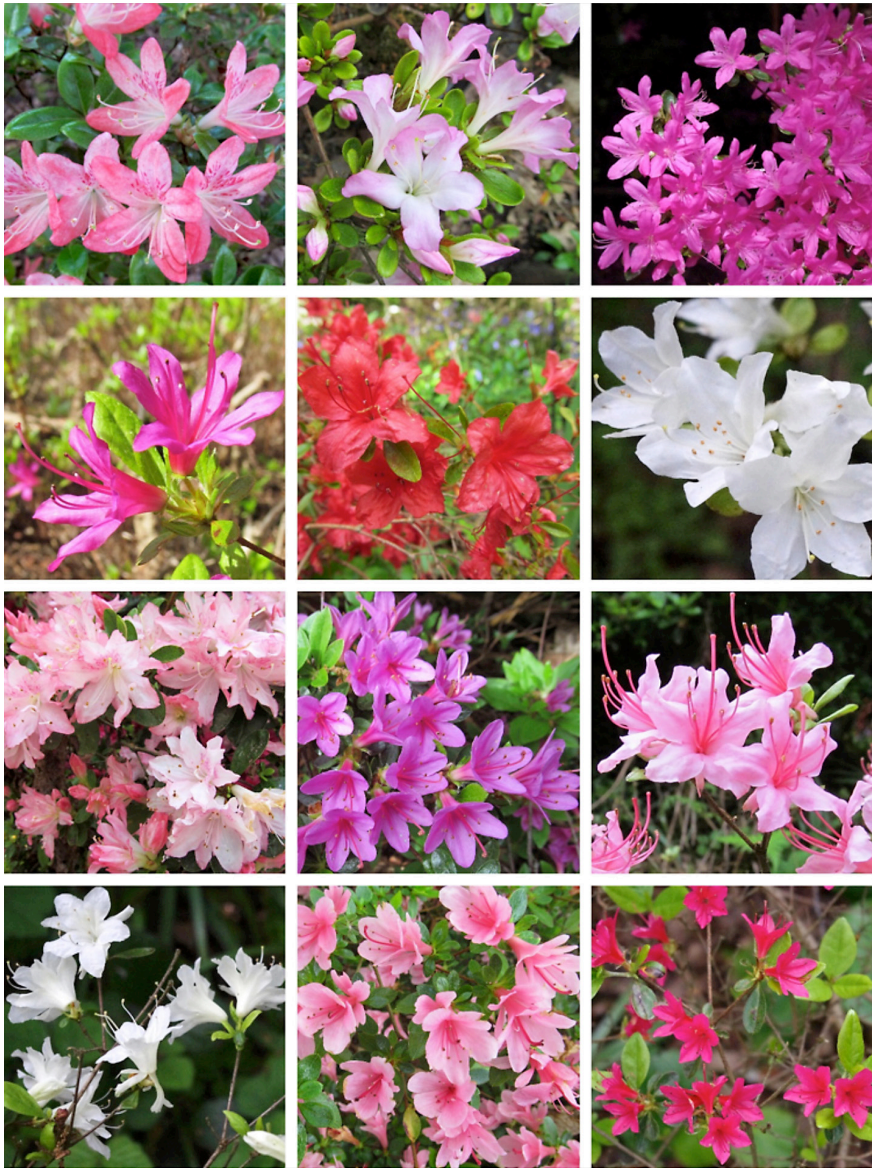
Figure 1. Twelve of the Kurume Azalea hybrid cultivars raised by Japanese plantsmen and imported to the West by Ernest Wilson. All are clones derived from natural hybrids between *R. kiusianum and R. kaempferi,* and illustrate the morphological variation produced by segregation. Top row (L-R): 'Asa-Gasumi', 'Shin utena', 'Saotome'. Second row: 'Katsura no Hana', 'Rasho Mon', 'Yoro Zuyo'. Third row: 'Azuma-Kagami'*, 'Kiritsubo', 'Shintoki no Hagasane'. Bottom row: 'Seikai'*, 'Kirin'*, 'Hinode no Taka'. Asterisks indicate double ("hose-in-hose") corollas, a condition not normal to either parent. All photos by author.

mediated superiority over their parents, because seed of pure parental plants would be produced in far greater numbers than F_1 seed due to the species barriers discussed above. Hence F_1s can only outnumber their parents if they have a fitness advantage, and the fact that they only do so within a specific habitat band indicates clearly that this advantage is habitat-mediated. Rare cases where F_1 advantage is not habitat-mediated, and F_1s therefore can invade parental ranges (e.g. *Typha × glauca*; Zapfe and Freeland 2015, Pieper *et al.* 2018) fall outside of the definition of F_1DZs.

F_1DZ formation requires superiority of F_1s over post-F_1s, as well as the parents. This could in theory be intrinsic (i.e., independent of habitat) or habitat-mediated, because either could explain the absence of post-F_1s from a population of fertile F_1s. Intrinsic fitness can be compared for seedlings using common garden trials (e.g., Criste *et al.* 2016). Theoretically, habitat-mediated fitness can be assessed using reciprocal transplants or culture under artificial habitat conditions for common garden trials. These run up against several problems, however. First, F_1DZs tend to occupy undisturbed habitats, whereas disturbance tends to favor post-F_1s, and so transplantations and artificial conditions will inevitably introduce confounding effects by increasing soil disturbance and potentially favoring post-F_1s. My own attempts to recreate soil conditions for hybrid class of *R. × intermedium* could not replicate wild conditions (Milne and Abbott 2008). Instead, only experiments where seeds are sown in natural habitats offer a realistic test for habitat-mediated fitness. A second problem is that the *Rhododendron* species involved tend to be long-lived, meaning that in most cases a meaningful experiment would take many years to complete. An exception so far may be with *Viola*, one herbaceous genus known to form extant F_1DZs (Table 2), for which the results of proposed transplant experiments are keenly awaited (Nagano *et al.* 2015). The practicalities of transplant experiments are further complicated by the remote nature of most F_1DZ sites, a consequence of these occurring on pristine natural habitats (Table 2). Finally, the rate of successful recruitment from *Rhododendron* seeds must be well under one in a million (see discussion below), and it may well be that, in most years, there is no natural recruitment at all in a stable population, unless the weather for recruitment is exactly right. If so, sowing experiments could easily yield zero survivors in a typical year, regardless of hybrid class or habitat. For now, direct evidence from common garden experiments concerns only intrinsic fitness (Criste *et al.* 2016), and the only evidence so far for habitat-mediated superiority of F_1s has been inferred by looking at hybrid zones between the same species, but in different conditions.

If F_1DZs are maintained by habitat-mediated selection against post-F_1s, then we would expect to see different hybrid zone compositions where the same species meet in different habitats, and this is exactly what is observed. The first detailed description of an F_1DZ, although they did not coin the term, was by Kyhos *et al.* (1981). Populations

of F_1 hybrids (*Encelia × laciniata*; Asteraceae) were found on leeward slopes of sand dunes and nowhere else, whereas in more disturbed habitats, hybrid swarms occurred, dominated by post-F_1s. In the Turkish *Rhododendron × sochadzeae*, F_1-dominated hybrid zones occupy a narrow altitudinal band separating the parent species, but a small hybrid population dominated by backcrosses was found lower down, in a steep-sided stream valley where late-lying snow apparently creates an unusual microclimate (Milne *et al.* 1999, 2003). In the case of the Chinese *R. × agastum*, the ecological separation between parents *R. delavayi* and *R. irroratum* is less clear. A role for the environment is implied because hybrid zones contain only F_1s at Hua Dian Ba near Dali (Zha *et al.* 2010) but form massive hybrid swarms at Baili (Marczewski *et al.* 2016). In all these three cases, therefore, F_1s thrive in habitats intermediate between those of the parents, while post-F_1s do better when disturbance or another factor has created a different habitat. This means that the full criteria for F_1DZs have been satisfied as given by Milne *et al.* (2003): F_1s dominate the hybrid zone with clear evidence that they are fertile, and that their superiority over other classes is habitat-mediated.

Another possible case concerns *Salvia × sylvestris*, which occurred with its parent species in undulating meadows south of Vienna, occupying sites of intermediate drainage (Kerner 1895). It was >60% fertile (Kerner 1895), and populations with many post-F_1s occur elsewhere. Unfortunately, this population appears to have been destroyed (R. Milne, pers. obs.), so we may now never know for sure.

Hybrid zones between *Rhododendron ferrugineum* and *R. hirsutum* in the European Alps satisfy all the above conditions, except that they contain 56% F_1s plus many backcrosses (but only to *R. hirsutum*). The parents are separated by their pH preferences, and hybrid zones (*Rhododendron × intermedium*) occur in mosaic habitats where soil types mix and pH varies dramatically on a small spatial scale (Milne and Abbott 2008). Hence F_1s might always outcompete other classes on soil patches with suitable pH, whereas different soil types support other classes. The exact proportion of F_1s varies between sites, again implying a controlling role for local habitat. Furthermore, in other sites where the role of human disturbance seems to be greater, the proportion of F_1s decreases (Bruni *et al.* 2016). Outside of *Rhododendron*, *Populus × canescens (= P. alba × P. tremula)* forms F_1DZs of between 56% and 85% F_1s in both Europe (Christe *et al.* 2016) and China (Zeng *et al.* 2016) (see Table 2).

Rhododendron aganniphum × R. phaeochrysum forms a population of fertile hybrids that do not intergrade with either parent, but the possibility has not been eliminated that these are a population of post-F_1 hybrids, stabilizing on their way to becoming a new hybrid species (Marczewski *et al.* 2015).

The last three known cases all involve large populations of F_1 hybrids in very

specific habitats, but for these no cases are known of hybrid zones elsewhere with a different structure, where post-F_1s dominate, and so the nature of their F_1 advantage is uncertain. Fertile *Phyllodoce* F_1s consistently dominate snowmelt gradients between the parent species (Kameyama and Kudo 2011). The *Viola* species *bissetii* and *rossii* occur on opposite-facing slopes, with F_1 hybrids dominating one ridge top between them (Nagano *et al.* 2015). F_1s of *Populus* × *jrtyschensis* occupy habitats that have lower nitrogen content than those of either parent (Jiang *et al.* 2016). This could mean F_1s here either prefer a non-intermediate habitat or that the habitat is intermediate in other unknown ways.

Had I myself not chanced upon the atypical hybrid zone at Çamlıhemşin, Turkey, during an earlier field trip (see Milne *et al.* 1999), I would have been far less sure about the importance of habitat for *R.* × *sochadzeae*. Future detection of hybrid swarms in any of the three cases above could show that F_1 advantage is habitat mediated, but it is far harder to prove that such swarms cannot form. Hence intrinsic F_1 advantage must instead be tested by common garden experiments across a range of conditions as Criste *et al.* (2016) have started to do.

Overall, these examples illustrate how F_1DZs tend to form where the habitats of the two parents grade into one another, creating a band of intermediate habitat in between. This habitat gradient may be altitude (*R.* × *sochadzeae*, *Phyllodoce*), pH (*R.* × *intermedium*), soil drainage and/or stability (*Encelia*, *Salvia* × *sylvestris*), or aspect (*Viola*). In other cases it is less clear, or in one case apparently transgressive (i.e., beyond the ecological tolerance of either parent, i.e., *Populus* × *jrtyschensis;* Jiang *et al.* 2016).

F_1DZs may have evolutionary significance as barriers to gene flow, if F_1 superiority prevents the recruitment of backcrosses (Milne *et al.* 2003), but clearly this effect won't be total unless all hybrids are F_1s. In many cases, a minority of backcrosses are present (Table 2), and Criste *et al.* (2016) have detected evidence of ancient introgression in *Populus* × *canescens*. Furthermore, in some cases there may be a strong barrier to backcrossing in one direction, but a much weaker one in the other, e.g., *Rhododendron* × *intermedium* (Milne and Abbott 2008). Hence F_1DZs might more accurately be said to restrict or minimize gene flow between species, relative to other forms of hybrid zone.

Why are F_1DZs so common in *Rhododendron*?

Among the F_1DZ examples above, three of the four most complete examples come from *Rhododendron*, plus one of the other six (Table 2). Detected F_1DZs are thus far more common in *Rhododendron* than in any other genus, with *Populus* coming a distant second. *Rhododendron* has also provided the only case I know of where F_2 (or other intermediate post-F_1) class hybrids outnumber backcrosses, in this case in a small

hybrid population between *R. cyanocarpum* and *R. delavayi* (Ma *et al.* 2010). What then makes *Rhododendron* so uniquely suited to producing these phenomena?

For an F_1DZ to form, the parents need to be highly interfertile, and there should be clear-cut ecological separation between them, leading to similar separation between F_1s, parents and other hybrid classes. This is true of *Rhododendron* but also countless other species pairs, so this can only be part of the explanation.

F_1DZ formation also requires that the effects of natural selection must be absolute: F_1s reach adulthood, and that other potential subsequent generations must fail to. Moreover, this has to happen despite F_1 seed presumably being produced far more rarely than both parental seed and, once a fertile F_1 is formed, post-F_1 seed. That no class other than F_1s reaches adulthood requires both (1) extreme selection, and (2) enough F_1 seed to populate the site. Regarding selection, F_1s should possess one complete set of genes from each parent, allowing them to thrive in environments that combine the conditions each parent is adapted to (Milne *et al.* 2003, 2004; Jiang *et al.* 2016). Conversely, segregation after the F_1 generation creates novel gene complexes and hence non-intermediate ecological needs in each post-F_1 individual (Anderson 1948, 1949), meaning that certain post-F_1s will outperform F_1s in habitats that impose novel, as opposed to intermediate conditions, e.g., habitat disturbance in *Encelia* (Kyhos *et al.* 1981). The same process is likely to break up co-adapted gene complexes that confer adaptation to stresses imposed by the habitat of each parent, however, reducing the ability of the F_2 to tolerate conditions in habitats intermediate between those of the parents (Milne *et al.* 2003, 2004; Jiang *et al.* 2016). This provides a mechanism for selection to strongly favor F_1s over post-F_1s in undisturbed, transitional habitats, but not atypical or disturbed environments. In theory, the break-up of gene complexes could also lead to lower fitness in post-F_1s than F_1s, the so-called "hybrid breakdown." This is likely to explain why F_1s seem to outperform other classes in *Typha* × *glauca* (Freeland *et al.* 2013), and possibly *Populus* × *canescens* (Criste *et al.* 2016). Segregation is likely to increase the variance of intrinsic fitness (Rieseberg and Carney 1998), however, meaning that if large numbers of F_2 seed are produced, a minority might be fitter than F_1s. This high variance might overcome hybrid breakdown in hybrid swarms, but would need to be weaker in effect if certain F_1DZs are maintained by intrinsic selection, as postulated above.

If selection for F_1s over other classes is strong enough, then an F_1DZ can form provided enough F_1 seed is available. Indeed, the actual proportion of F_1s among seed that reaches the site should not matter, only that enough of it reaches the site to populate it. In some cases, F_1 might be the only seed that is even capable of forming adult plants on sites where F_1DZs form. Given that the total amount of F_1 seed produced by the parents is a product of the overall number of seeds produced by the

parents, and the proportion of that seed that is F_1, it follows that the likelihood of F_1DZ formation increases with the quantity of seed produced in the species involved. Cross (1975) estimated that a large, mature *Rhododendron ponticum* can produce around a million seeds per year. They start flowering after 12 years and live for >100 years (Cross 1975), so could have a lifetime output of millions of seeds. Only two of these seeds need to survive to maintain the population. If extreme selection for F_1 seed occurs at seed germination and the seedling stages, enough surviving F_1 seed could then dominate suitable habitats. Hence the production of extremely high numbers of seeds, permitting selection to act with high intensity upon them, might be a vital component for F_1DZ formation, and a significant part of why it happens more in *Rhododendron* than in any other genus. Among other cases of F_1DZs, *Populus* can also produce seeds in comparable numbers to *Rhododendron*, but less so for *Encelia* and *Phyllodoce*, and especially *Viola*. Possibly clonal spread and long lifespans may play a role in these latter cases.

Hiding in plain sight: F_1DZs masquerade as species.

F_1DZs have only been understood fairly recently, compared for example to Anderson's (1948, 1949) detailed descriptions of hybrid swarms and the effects of segregation, and other models that describe hybrid zones (e.g., Moore 1977, Barton and Hewitt 1985, Harrison and Rand 1989). This may be in part because F_1DZs do not look like other hybrid zones: a population of segregating diverse hybrids is unmistakable because of its morphological diversity, but paradoxically F_1 hybrids may all look similar. Thus, when a biologist sees a large number of morphologically similar F_1 individuals together, with few or no intermediates to other taxa nearby, the initial conclusion will be that they are a species. Of the ten cases listed in Table 2, *Salvia × sylvestris* and *Rhododendron × intermedium* were both taken for stabilized hybrid species by Kerner (1895), whereas *R. × sochadzeae, R. × agastum, Encelia × laciniata* and *Phyllodoce caerulea × aleutica* were all initially described as species or non-hybrid taxa.

Based on this, F_1DZs probably exist that have not yet been diagnosed, and some may even have been described as species. Any supposed "species" that is brought into cultivation can be identified as an F_1 as it won't breed true, so this can identify some uncertain *Rhododendron* "species" as being possible F_1DZs. Because so many examples of F_1DZs come from the genus *Rhododendron*, however, we should nonetheless expect that more F_1DZs may exist in this genus, waiting to be detected.

British *Rhododendron ponticum*: Is it a hybrid swarm?

Considerable confusion has arisen recently concerning the exact nature of naturalized British populations of *R. ponticum*. Without doubt, some of the British material is introgressed with genetic material of *R. catawbiense, R. maximum* and very probably

R. arboreum, though not *R. macrophyllum* (Milne and Abbott 2000), and some other species might be involved on a highly local basis (Cullen 2011). Cullen's (2011) reference to this material as a hybrid swarm has led, however, to considerable confusion, and his attempt to reclassify all British material as *R. × superponticum* is unsupportable. It is necessary, therefore, to clear up some confusion here.

Both Cullen's (2011) meticulous morphological investigations, and my own (Milne and Abbott 2000), indicate that some, but not all, British *R. ponticum* plants show evidence of introgression. The vigor of British material is very probably not, however, due to introgression (Erfmeier *et al.* 2011). Hence, there is no morphological trait upon which British material can be consistently distinguished from *R. ponticum* elsewhere, and some of it is almost certainly genetically indistinguishable from the native Iberian material.

R. ponticum entered the British countryside through numerous deliberate plantings in the 19th Century, plus countless localized escapes from gardens and estates ever since (Milne 2017). Each planted population has its own unique history in cultivation, and in each case that history might involve hybridization with one other species (most commonly *R. catawbiense*), or many species, or none at all. Furthermore, ancestry will vary even within a population, especially where crossing has occurred with less commonly grown species in local plantings. Given this highly complex situation, applying a single hybrid name is completely untenable, either to all British material, or even to the minority that shows evidence of introgression. In short, the name *R. × superponticum* should be avoided entirely.

Cullen's (2011) described British material of *R. ponticum* as a "hybrid swarm." Whether or not this was technically correct is debatable, and it is likely at best a gross oversimplification. Though Cullen's (2011) paper of course gives far more detail, problems are being created by others when they repeat the "hybrid swarm" description, causing multiple misconceptions by anyone not familiar with the detailed history of this plant in Britain. The term "hybrid swarm" here is misleading for the following reasons. First, variation in British plants is due to varying levels of introgression from different species, whereas applying the term hybrid swarm implies that it is due to segregation. Second, the term wrongly implies that hybridization is ongoing, when most or all hybridization happened in cultivation before the plants escaped. Third, it implies that all material is hybrid when this is almost certainly untrue. Fourth, it implies that all British plants form one interconnected entity. Hybrid swarms can sometimes involve more than two species, but in British *R. ponticum* not all individuals have the same ancestry, as each population derives from a local planting, and there may be over a dozen hybrid combinations present. Finally, "hybrid swarm" greatly overstates the influence of hybridization, implying that British material might typically only be

around 50% *R. ponticum*, when in fact it is probably 90 to 95% *R. ponticum* (Milne and Abbott 2000).

Overall therefore, British material represents an exceptionally complex situation, with varying levels of influence from an unknown number of other species. Any attempt to subdivide this material taxonomically is doomed to failure, and better understanding will be therefore achieved if the term "hybrid swarm" is avoided. Instead, it should simply be referred to as genetically altered *R. ponticum*.

Conclusion

Rhododendron hybrid zones offer fertile grounds for scientific research because they often display very unusual characteristics, such as the relative frequency of F_1-dominated hybrid zones, but also one curious case where F_2s seem to be the most common class. To study this further requires understanding of hybrid fitness, but the long lifespan of rhododendrons makes it difficult to study hybrid fitness empirically, by making transplant and common garden experiments to test hybrid fitness difficult to achieve.

Formation of F_1DZs always requires habitat-mediated superiority of F_1s over parents, as the precise habitat zonation of F_1s in all cases in Table 2 cannot be explained in any other way. They also require that adequate quantities of F_1 seed are produced, which in turn requires two things to be high: seed set and rate of hybridization. That it satisfies these requirements so well might be what makes F_1DZ formation more common in *Rhododendron* then elsewhere. There is less certainty over why there is F_1 superiority over post-F_1s. In *Rhododendron* and *Encelia,* this seems to be habitat mediated, whereas in *Populus × canescens* and possibly *Phyllodoce,* it is probably not related to habitat. The situation with other species needs more data to resolve. Of course, both explanations might apply in some cases, and the question might be better put as whether the habitat dependent or independent component of F_1 advantage has the greater effect.

Although only briefly touched on here, the potential role of hybridisation and hybrid speciation in the evolution and diversification of the genus could be significant, and ironically one of the biggest obstacles to unpicking these effects may be the occurrence of regular introgression between extant species, making the signature of ancient hybridization events difficult to detect. With all these unique aspects, *Rhododendron* is thus an excellent model for examining the consequences of natural hybridization

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Where Senses and Science Converge

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Introduction

As the minority horticultural viewpoint in this issue, I am using my page allotment to discuss common interests shared by gardeners and researchers and how garden clubs like the American Rhododendron Society (ARS) and the Azalea Society of America (ASA) have facilitated scientific inquiry. The key connection here is that plant societies consist of plant enthusiasts who are curious to know more about the beautiful plants that they enjoy at home, who have the desire to study species, to explore plants in the wild, to test clones in different environments, to establish collections, and to improve the cultural techniques for growing rhododendrons and azaleas. At the same time, a scholarly interest among some members has resulted in ARS/ASA journal articles, bibliographic services, databases, and archives that are available in print, CDs, or online. These endeavors have great value for science, but most plant scientists don't interact with or appreciate the wealth of knowledge that garden clubs have to offer research. Many of these activities are described in more detail on the ARS website, www.rhododendron.org; the ASA website, www.azaleas.org; and the first two issues of this journal (*Rhododendrons International*) which describe the worldwide interest in *Rhododendron* among gardeners, www.rhododendron.org/ri-index.htm. Some examples of how ARS/ASA endeavors facilitate scientific inquiry are given below.

Rhododendron collections with horticultural roots

1) Rhododendron Species Botanical Garden, Federal Way, WA, USA: Growing public interest in rhododendrons led the ARS to form the Species Project in 1962, chaired by Dr. Milton Walker, who was instrumental in arranging a shipment of

rhododendron and azalea species cuttings from the U.K. to Canada for propagation and eventual distribution to the U.S., where they were initially grown on Dr. Walker's estate. Subsequent need for more space and financial support resulted in the establishment of the Rhododendron Species Foundation (now Rhododendron Species Botanical Garden), created by the ARS board as an independent non-profit membership organization dedicated to the conservation, public display, and distribution of Rhododendron species (Hootman 2016). Home to one of the largest collections of species *Rhododendrons* in the world, the garden displays over 700 of the more than 1000 species found in the wilds of North America, Europe, and Asia (rhodygarden. org/cms/).   [Editor's note: In this issue, Medeiros *et al.* provide an overview of some research taking place at this garden.]

2) Rhododendron-Park, Bremen, Germany: This collection was founded in 1936 by German Rhododendron Society in partnership with the city of Bremen municipal parks department and local *Rhododendron* industry (Schepker 2016). The park has expanded from its original two to 46 ha (five to 114 acres) that showcase one of the largest *Rhododendron* collections in the world with over 550 species and 2500 cultivars (rhododendronpark-bremen.de/). The society remains actively involved with the Rhododendron Park and supports regional research on *Rhododendron* taxonomy, plant breeding, culture, databases, and species exploration. [Editor's note: In this issue, Khunert *et al.* provide an overview of some research taking place at this garden.]

Many other *Rhododendron* collections worldwide are maintained at public arboretums or botanical gardens, and some of these are supported by manual labor, expertise, and financial help from local rhododendron and azalea clubs. The importance of these collections cannot be understated. They are essential for *ex situ* species conservation and for easy access to and exchange of plants that naturally occur in remote locations. The taxa are usually arranged and displayed in a manner that enhances public enjoyment and education. Collections of species and cultivars also provide the raw material for plant breeding, which is focused on recombining traits in novel hybrids. And because collections in different locations often overlap, containing genetically related and sometimes identical (clonal) accessions, they can be used to study the interactions of genes and environments, such as the effects of climate on plant phenology or identifying adaptive limitations among taxa.

Research funding
1) Rhododendron Research Foundation: The RRF was formed in 1976 as a trust agreement between a group of trustees and the ARS. It is operationally and financially independent of ARS, soliciting and investing donations for its continued support of research. Rhododendron and azalea research proposals that are theoretical or applied

can be funded for up to $5000, a level that is useful for doing exploratory research or pursuing questions unique to the genus that might not be funded by larger institutional grants. The grant guidelines list research topics that are currently priorities for the ARS, and the list is updated periodically (www.rhododendron.org/researchgrants.htm). To date, over 144 projects have been funded, with examples of scope given below:

Research Foundation Funding as of 2008

Research Category	No. Proposals Funded
Botanical research	17
Propagation and culture	14
Control of diseases and pests	27
Physiology/Stress tolerance	21
Genetics & cytology	19
Collections and conservation	10
Plant culture	16
Plant breeding research	7
Research archives and database	4
Other	7

http://scholar.lib.vt.edu/ejournals/JARS/v62n1/v62n1-bernady.htm

2) Azalea Research Fund: This fund was started in 2009 by the ASA at the suggestion of Dr. August Kehr, a former United States Department of Agriculture scientist and ornamental plant enthusiast. Its mission is to support research that will "foster increased knowledge and improvement of the standards of excellence with regards to azaleas." The level of funding and scope of research priorities are similar to those of the RSF— www.azaleas.org/research/arf-research/.

Seed exchanges

Both ARS and ASA have rhododendron and azaleas seed exchanges that are supplied and used by members (who get first pick), as well as non-members. Other international rhododendron societies provide a similar service for their members. For decades, this has been one of the most popular and efficient means of making the global diversity of *Rhododendron* available to all. Some of the seed is wild-collected (species), some from open-pollinated clones in member gardens, while most is seed from hybridizers who are making controlled crosses between different species or cultivars to recombine traits. In addition to the personal enjoyment of growing plants from seed to flower, participants are guaranteed genetically unique plants that may have novel features. Contributors are

encouraged to provide accurate and detailed information about the seed source. The cost is nominal, about $2 (US) for 50 seeds plus shipping. The links are: www.rhododendron.org/seedexchange.htm (ARS) and www.azaleas.org/2018-azalea-seed-list/ (ASA).

Reference materials

1) Digital journal archives: The *Quarterly Bulletin of The American Rhododendron Society* was first published in 1947 and evolved into the *Journal American Rhododendron Society* (JARS) in 1982. *The Azalean*, a quarterly published by the ASA, was first issued in 1979. Both journals include contributions from and provide a wealth of information for enthusiasts, educators, and researchers. The research articles that appear from time to time are peer-reviewed but the journals are not included in scientific citation references such as ISI. Nonetheless, there are some very good data that appear in these issues. For example, one can currently find more information about *Rhododendron* pH tolerance in JARS than currently exists among mainstream plant journals. The ARS Digital Publications Committee, spearheaded by members Herb Spady and Bob Weissman, has produced electronic issues of JARS from 1947 to 2008 that are freely available on the ARS website. While the digitizing process is still underway, the objective is to have all issues but for the latest ongoing five years (most recent 20 issues) in the database. The latter are not included, as their access is restricted, and is one of the benefits of having ARS membership. The digitized journal is archived at the Digital Library and Archive at Virginia Tech University and can be Google searched by keyword(s)—http://scholar.lib.vt.edu/ejournals/JARS/. It should be noted that a very few article authors have specifically requested that their articles not be made available in the Digital Library and Archive at Virginia Tech University, as they would like the opportunity to make that information available in books they may publish, so the digital JARS archive is not totally complete. Nevertheless, this is a tremendous asset for accessing over 60 years of wide-ranging articles on rhododendrons and azaleas. Starting in 2012, JARS also became available in both paper and digital formats, and the digital format can be accessed by ARS members with their passwords at https://www.arsoffice.org/protect1/users/online.asp. It should be noted that formats, and hence article page numbers, differ between the print and digital formats.

Back issues of *The Azalean* are also available and searchable online at the ASA website, although a member password is needed to access the most recent 12 issues.

2) Rhododendron hybrids – a guide to their origins: This useful book was first published in 1986 by nurseryman Harold Greer and university professor Homer Salley, both members of the ARS. The project received multiple funding cycles from the Rhododendron Research Foundation, expanding to two volumes and a companion CD.

The volumes trace the ancestry of thousands of hybrid rhododendrons through one or more generations to the original species from which they derived. This is made possible due to the early-19th century practice of pedigree breeding in rhododendrons, where selected individuals from each generation are mated to produce the next. Compared to most ornamental plants, rhododendron cultivars are genetically complex because they often result from interspecific hybridization between several different species over multiple generations. Sally and Greer's (1986) book is used by many hybridizers who want to know more about the genetic backgrounds of plants that they are considering as parents for breeding. The pedigrees also have a number of scientific applications. They can be used to calculate consanguinity (relatedness) and the effects of inbreeding on plant traits. Genealogies can also be used to study trait inheritance in family lineages, as is frequently done in human genetics. However, most plant geneticists prefer to make their own structured populations via selfing, sibling, or parent-offspring matings (e.g., F_2 or backcross generations), which result in large segregating populations that cannot be achieved in human research due to practical and ethical concerns.

3) ARS website 'Plant Data.' This website tab can also be used to query pedigree information on specific cultivars, or on groups of plants sharing common traits or origin (hybridizer) using a search engine. This can be used to conduct armchair investigations, such as asking the question "is yellow flower color in rhododendrons maternally inherited?" This is an oft-debated issue among hybridizers. Going to the "Plant Data" tab on the ARS home page, selecting "Rhododendrons," and typing "yellow" in the flower color field produces a list of hybrids that meet that description. By clicking on the "more info" link, one can ascertain the parentage for each cultivar (the answer is that the yellow flower trait in progeny can derive from either the pollen or seed parent, so it is likely to be an autosomal trait). While the Rhododendron Hybrids CD provides more in depth information, it appears to be more limited in search capabilities

4) Rhododendron and Azalea Research: This is a hardcopy database of 1000 citations with brief abstracts (Salley 1992). The project was funded by the Rhododendron Research Foundation. A second volume was later produced (Salley 1994) which added 800 more citations of scientific writing on genus *Rhododendron* through 1993. Collectively, articles from 544 authors appearing in 461 journals are organized into broad topic areas and are searchable by author and/or subject indices. A list of journals cited is also included in each volume. Dr. Salley's work complements a bibliography published by Hannover University, Germany (Spethmann et al. 1992, 1996, 2002).

5) The International Rhododendron Register and Checklist, second edition, 2004: This two-volume set, now with seven supplements, was compiled by Alan Leslie, the recently retired International Rhododendron Registrar, and published by the Royal Horticultural Society. It contains the most up-to-date listing of all rhododendron

hybrids, with over 28,000 entries of both registered and unregistered names for cultivars and Group epithets (including their synonyms), as well as the names of nearly 2000 individuals or firms that have played a role in the raising, naming, introduction or registration of these plants. The second edition covers all azaleas, azaleodendrons, vireyas and "hardy" rhododendrons. It uses the botanical nomenclature and classification followed in reporting on parentage and origin of the cultivars and Groups based on the revision of the genus *Rhododendron* pioneered by David Chamberlain and James Cullen, published in 1996. The one exception is with *R. yukushimanum*, which was retained even through it has been revised to be a subspecies of *R. degronianum*. The hard copy of this set is difficult to obtain, but it and its supplements have just recently been released in digital form (International Rhododendron Register Second Edition Single Volume Edition FOR WEBSITE.pdf, 119 mb) by Pam Hayward, Rhododendrons, Camellias and Magnolias Group, RHS, which has made it much easier to both obtain and search for entries. It really is an essential document for anyone interested in searching out what *Rhododendron* cultivar names have been adopted, and what are the parents and characteristic features of each accepted specific cultivar and Group name.

Species in the wild

Rhododendron enthusiasts who grow species and hybrids in their gardens appreciate what nature has to offer and are supportive of their conservation efforts both *in situ* (in the wild) and *ex situ* (in culture). In North America, efforts in the field occur mainly on the east and west coasts where most of the native rhododendron and azalea species grow. Fortunately, in the USA, many of the ranges of these Rhododendron are distributed on federal or state protected lands. Some state-listed, rare species in the American Midwest have received special protection, such as the Rhododendron Cove State Nature Preserve in Ohio, which contains disjunct populations of the rosebay rhododendron (*R. maximum*) and flame azalea (*R. calendulaceum*).

Gregory Bald: Many summits in the Smokey Mountains of North Carolina are forested, but others have open meadows called "balds." Gregory Bald is noteworthy because it contains several native deciduous azalea species that bloom spectacularly in June, surrounded by broad mountain vistas at 1845 m (5000 ft) elevation. The high diversity of flower color and form is thought to result from natural hybridization between four azalea species that are sympatric at that location—*R. arborescens, R. calendulaceum, R. cumberlandense*, and *R. viscosum.* The origins of the balds (causes of deforestation) are obscure, but it is generally agreed that without current periodic clearing, the meadows would become overgrown in a short time. To prevent this from happening, a partnership was formed between the Mid Atlantic Chapter (MAC) of the ARS and the National Park Service in which ARS funding was used both to purchase a

mower and to hire an intern to reestablish the site to its "original" 33 open acres (13.5 ha). The effort was led by MAC members Don Hyatt, Jim Brandt, George McLellan, and Sandra McDonald, who have made the study and preservation of native azaleas in the wild a lifetime pursuit.

Western North American Rhododendron Species Project (WNARSP) http://www.wnarsp.org/index.html: The WNARSP initiative started among a group of *Rhododendron* species enthusiasts in Washington State, and has operated under the auspices of the Rhododendron Species Botanical Garden in Federal Way, WA. The goal was to compile location information about their native species populations from a variety of sources, including local naturalists, universities, and government agencies. This has resulted in species maps for the following taxa: *R. occidentale, R. albiflorum, R. camtschaticum, R. groenlandicum, R. macrophyllum, R. neoglandulosum,* and *R. tomentosum,* that collectively range from the Arctic Circle to southern California near the border with Mexico. The location data has been digitally archived and was used to generate interactive maps of known indigenous populations. According to their homepage, WNARSP "endeavors to spread the knowledge through their website, magazine and newsletter articles, and custom maps for researchers." This fine scale information could prove useful for conservation efforts or studies of local adaptation in taxa that occur in a wide range of habitats. Further inquiries about this database can be made through the Rhododendron Species Botanical Garden, wnarsp@rhodygarden.org.

Future roles for plant societies

The examples above illustrate how plant societies can lend their organizational weight to promote further interest in and knowledge about their beloved subjects. Equally evident is a long history of individual volunteers within the general membership who have taken on big projects and used their talents and time to produce substantive work. However, as with many other gardening societies, membership in ARS has decreased from its high point of 5600 (1994) to about 2400 members in 2018. While rhododendrons and azaleas remain commercially important landscape plants, many in future generations that might enjoy a few shrubs in their gardens may be less inclined to develop extensive and diverse home collections, and with so much information now on the web, furthering their plant knowledge in the company of like-minded club members may not be as attractive an option. To offset these trends, rhododendron societies world-wide could benefit from greater collaboration among them and the development of projects and programs that attract a global and perhaps more web-oriented audience.

In addition to shifting social preferences, the forces of global trade and climate change are presenting new challenges for the cultivation of all ornamental plants, but in particular for taxa such as *Rhododendron* that are not broadly adapted to a wide range

of growing conditions. The advent of higher temperatures combined with changes in precipitation (drought or flooding) will undoubtedly cause excessive stresses both in garden landscapes and in the wild. These abiotic stresses can also modulate biotic stresses as, for example, increases in temperature and rainfall increase the range and disease pressure exerted by plant pathogens. In addition, the rapid introduction of non-native pests and diseases via globalization constitutes a severe biodiversity threat to organisms that have not experienced them before. These problems are not unique to *Rhododendron*, but the high level of diversity within the genus offers the possibility that natural variation in adaptive traits might mitigate some of the effects of climate change and invasive species. From a horticultural perspective, it will be increasingly important to identify functional diversity in the wild for breeding new varieties that can better adapt to stressful environments and be successfully grown by consumers. Maintaining the public popularity and commercial viability of rhododendrons and azaleas is important to the future of plant associations like both the ARS and ASA, because having a beautiful and healthy plant at home is often the first step towards deeper knowledge and engagement.

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Monitoring *Rhododendron* Flowering Times in a Changing Climate

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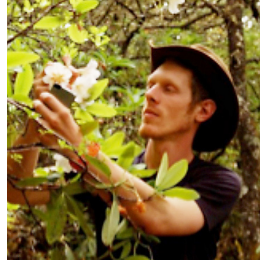
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Introduction

Phenology—the seasonal timing of life history events—is a critical dimension of plant biology. It affects growth, reproductive fitness, natural hybridization patterns, the survival of individual plants and the persistence of entire populations. In rhododendrons, flowering phenology can be as dramatic a moment in natural settings as it is in gardens. The flowering of rhododendrons in the eastern Himalaya—where the genus' species are at their most diverse—was described by plant hunter Frank Kingdon-Ward in 1926 as "a tidal wave of tense colours which gleam and glow in leagues of breaking light" (Ward *et al.* 2008 [1926].

These displays have also made *Rhododendron* flowering an important moment in the seasonal rounds of native Himalayan peoples. For example, the blooms of *Rhododendron* signal to high-elevation indigenous Himalayans that the time has come to begin agricultural activities. In the western Himalaya, local festivals such as flower day (*phool sankranti*) from mid-March until April celebrate this onset of spring; similar spring festivals are celebrated in the eastern Himalaya in various forms where *Rhododendron* blooms presage the arrival of spring (Ranjitkar *et al.* 2014a).

The flowering also represents an important source of food for pollinators, including insects and birds (Bawa and Kadur 2013; Huang *et al.* 2017). The wide extent of *Rhododendron* flowers across season and elevation provides a continuous food source

for these animals. Where one species is spread along an elevational gradient—such as *R. arboreum* in Nepal—pollinator populations may build up first at lower elevations and later be able to move to higher elevations as availability of pollen at the lower locations decreases (Ranjitkar *et al.* 2012). At higher elevation, species-rich sites, the staggered phenological progression of flowering across the season as one species gives way to the next establishes continued provisioning to pollinators and other floral visitors (Hart and Salick 2018).

Shifts in phenology are one of the most obvious ways in which plants respond to climate changes. Across the globe, both long-term and wide-scale records of plant reproductive phenology are being associated with climate change. These range from historical records of the seasonal timing of cherry-blossom flowering in Japan (Aono and Kazui 2008), to the wine-grape harvest in France (Cook and Wolkovich 2016), the records of amateur naturalists recording plant flowering times in England (Fitter 2002) and America (Miller-Rushing and Primack 2008) and contemporary scientific phenological observations by, for example, the USA National Phenology Network, the Chinese Phenological Observation Network (Ge *et al.* 2014) and the Pan European Phenology Project (Basler 2016).

When plant phenology functions as an indicator of other seasonal events, shifts in plant phenology can be culturally salient signs of broad scale responses to climate effects across the environment. For example, *rhododendron* blooming is very closely observed in Kathmandu and associated with a local festival called *lhuti punhi*. In recent years, both locals and pilgrims have reported that the *rhododendron* bloom has completed already by the dates when this festival is celebrated. Similar changes in seasonal indicators have been used to infer climate change at sites across the globe. These include observations by indigenous people (Lantz and Turner 2003, Nabhan 2010, Armatas *et al.* 2016), by citizen science networks (Lawrence 2009), and in historical contexts where instrumental observations of climate events are lacking (Chuine *et al.* 2004).

Of course, shifts in phenology are also important in and of themselves. Although plant reproductive phenology generally advances with warming temperatures (Ellwood *et al.* 2013, Cleland *et al.* 2007), certain species may not have sufficient capacity to make the behavioral or evolutionary changes necessary to keep pace with a changing climate (Cook *et al.* 2012, Visser and Both 2005). Mechanistically, species respond to different environmental signals, such as growing season temperatures, day length, snowmelt timing, and chilling requirements (Keller and Körner 2014, Lamber *et al.* 2010, Schwartz and Hanes 2010, Clark *et al.* 2013). In the context of phenological progressions, differential responses may lead to timing mismatches among plant populations or among plant-animal communities, including pollinators or herbivores (Parmesan 2006; Post 2008). Even when a phenological shift in response to temperature

cues is adaptive, it can also expose a species to new threats, including novel temperature, water, or pest conditions.

Here, we review the results of recent research by scientists, including us and our colleagues, on how *Rhododendron* flowering times respond to climate change. We focus particularly on results from our fieldwork in the eastern Himalayas, and conclude with some thoughts about the potential benefits of a network of *Rhododendron* scientists and growers to collect uniquely relevant data on the phenological responses of common species to varying climatic conditions.

Rhododendrons of the Eastern Himalayas

The eastern Himalaya and associated Hengduan Mountains of China are among the world's richest areas for temperate plant species (Mittermeier *et al.* 2005, Mutke and Barthlott 2005), and the region is particularly rich in rhododendrons. Out of the perhaps 1000 *Rhododendron* species in the world, 30-50% are found in this area and many are endemic to it, being found nowhere else (Wu *et al.* 1996). *Rhododendron* species richness is particularly great in the area that contains extreme north-eastern Myanmar, the southeastern section of the Tibetan Autonomous Region (Xizang Province) and adjoining parts of Arunachal Pradesh, southwestern Sichuan Province, and northwestern Yunnan Province (Figure 1). Here, biogeographic connectivity, topographic heterogeneity, and climate seasonality have all been suggested to drive this richness (Wen *et al.* 2014, Shrestha *et al.* 2017). Using data from the *Atlas of Woody*
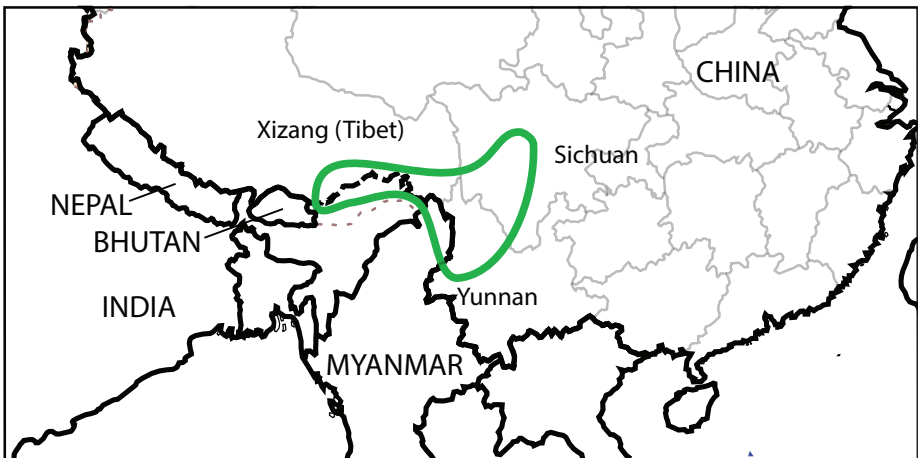

Figure 1. *Rhododendron* species are particularly rich in the eastern Himalayan and Hengduan region, where a relatively small area holds hundreds of species. The green area indicates the region where Shrestha *et al*. 2017 document >100 species / 50x50 km grid cell.

Plants in China (Fang *et al.* 2011), Shrestha *et al.* (2017) estimated *Rhododendron* species richness in 50 x 50 km grid cells and found that it frequently exceeded 100 *Rhododendron* species *in a single grid cell*. The eastern Himalaya also constitute a highly threatened area for rhododendrons. Yu *et al.* (2017) described 12 general hotspots for rhododendrons based on 10 x 10 km grid spatial analysis. They recognized five hotspots of threatened species: in southern Chongqing, south-eastern Tibet, north-western Yunnan, south-western Sichuan and northern Guangdong. These largely overlap with the general eastern Himalayan/Hengduan hotspot.

In addition to the effects of climate on *Rhododendron* flowering time, there are also effects on where rhododendrons are able to grow. With ongoing warming, we can predict future changes in the geographic range of suitable climate for Himalayan across the Himalayas (Figure 2). The shift northwards and up in elevation that this predicts is borne out by *Rhododendron* shrub encroachment up mountain slopes and into mountain meadows, which can be quantified from remote sensing (Brandt *et al.* 2013). However, although rhododendrons may respond to climate change with changes in phenology on an annual basis, as woody and relatively long-lived species, their ability
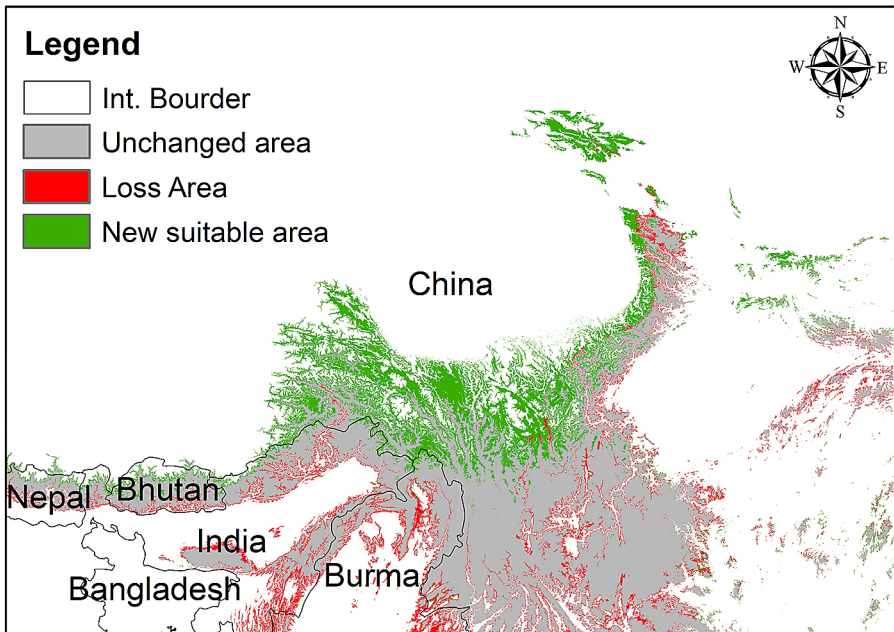

Figure 2. Shift of the bioclimatic suitability for rhododendrons in a climate warming scenario (the 2050 IPCC5 scenarios ensembled layer for all the models for RCP 2.6, compared to baseline climate 1960-2000).

to change geographic ranges must be much slower. In fact, it has been suggested that current Himalayan *Rhododendron* populations at low elevations may be remnants from larger populations present during a cooler period 150–500 years ago (Vetaas, 2002).

In addition to being exceptionally rich in *Rhododendron* species, the eastern Himalaya are also extraordinarily well-represented with historical collections of rhododendrons, thanks to plant hunters including Frank Kingdon Ward (quoted above), as well as Jean Marie Delavayi, Heinrich Handel-Mazzetti, George Forrest, Joseph Rock, George Ludlow and Frank Sheriff, Yu Dejun and Feng Goumei. Many of the species rhododendrons and parents of the hybrids now in cultivation stem from seed collections made by these botanists. The voucher specimens—dried flowers with associated labels data on the time and place of collection—that these collectors made remain housed in herbaria around the world (Hart *et al.* 2014).

Of course, indigenous Himalayan peoples had been encountering rhododendrons long before these botanists arrived. Throughout the Himalaya, rhododendrons have been objects of both utilitarian value (including for craft, firewood, food and medicine) and cultural significance (including as a symbol in religion and art, and as with growers around the world, a source of aesthetic pleasure; Georgian and Emshwiller 2013, Hart and Salick 2017, Kunwar *et al.* 2006).

Rhododendrons as an indicator of Himalayan climate

Although phenological events—especially those in the spring—generally *advance* in date with warming temperatures, moving earlier into the spring, this is not especially evident in the Himalayan and Tibetan Plateau region (Klein *et al.* 2014). This is despite the fact that this area has been warming, and is modeled to continue to show increases in temperature 1.5-1.75 times greater than the global mean increase (IPCC 2013). In 2009-2013, we set out to use focused, cross-disciplinary studies of *Rhododendron* phenology to better understand how changes in the Himalayan climate were affecting Himalayan plants. We expected that rhododendrons, with 1) an array of species flowering across elevations and throughout the season, 2) visually salient flowers, and 3) an excellent record in natural history collections, would be an excellent study system to leverage traditional ecological transect methods against (Hart and Salick 2018), to obtain anecdotal information through interviews with indigenous people (Hart and Salick 2017) and for quantitative analysis of relevant data from herbarium collections (Hart *et al.* 2014).

Himalayan rhododendron phenology and ecological monitoring

To gain direct observational and experimental understandings of how *Rhododendron* phenology is structured and may respond to climate change, we tracked flowering

over two years in an assemblage of ten co-occurring Himalayan *Rhododendron* species (Figure 3) on Mt. Yulong in Northwest Yunnan (Hart and Salick 2018). Our monitoring spanned an elevation range of 2600-4200 m above sea level, and flowering times from February to August. We also moved plants of two *Rhododendron* species, *R. hippophaeoides* and *R. racemosum*, into a greenhouse to artificially increase air temperatures. Across the elevational transects, we monitored rhododendrons on several transects every 100 meters of elevation by tagging twelve plants of each species present, and counting the number of inflorescences on each plant budding, in open flower, with finished flowers, and with mature fruits twice-monthly.

Overall, we observed advanced phenology in response to warmer temperatures, with earlier flowering at lower elevations (or, ~5 days later with each 100 meters up in elevation), in the warmer year (~2.5 days earlier per 1° C annual average temperature increase) and in the greenhouse treatments (~9 days earlier per 1° C difference in annual average temperature). We observed similar trends further west at Mt. Kanchenjunga in Nepal, where flowering of *R. arboreum* was 4.3-5.8 days difference per 100 m elevation (Ranjitkar et al. 2012). However, within this broad pattern of elevation-dependent phenology, other environmental and habitat qualities also had significant impacts on flowering time. Among the diverse species monitored on Mt. Yulong, the degree and even direction of phenological response to temperature varied. At the level of individual plants, there were clear fitness implications of the phenological shift: plants that responded to annual temperature increases by flowering modestly earlier with the warmer temperatures had the greatest reproductive success.

We were also able to test the apparently sequenced progression of flowering in the Mt. Yulong *Rhododendron* assemblage, and found evidence that there was indeed less overlap in flowering times in co-occurring *Rhododendron* species than expected by chance. Recently, a similar effect has been recorded in an assemblage of *Rhododendron* species in Sikkim (S. Bassnett, pers. comm. 2018). Given differential responses to changing climate, this suggests that hybrid barriers, floral visitor partitioning, or other functions of sequenced progressions may be vulnerable to ongoing climate change.

Himalayan *Rhododendron* phenology: local knowledge

Even given these differential responses among species, we might expect the clear and consistent effects of warmer temperatures on flowering would have resulted in gradual earlier flowering over time in the genus as a whole. Temperatures in the Mt. Yulong area, as in other areas across the eastern Himalaya, have been warming for the last 60 years, and at a faster rate for the last 30 years or so (Hart *et al.* 2014). In order to gain a longer-term perspective than our two-year ecological monitoring study could offer, we conducted interviews with rural village residents of Naxi and Yi ethnicities who

live around Mt. Yulong. We began with the hypothesis that the sequential and visually salient flowering of rhododendrons would have a place in local culture, and that local people would notice and report its phenology responses to a changing climate. Using photographs of *Rhododendron* species we asked 80 respondents in eight villages about *Rhododendron* species identities, names, uses, flowering times and their elevation distributions, and the changes in *Rhododendron* characteristics that they noticed over time.

The interviews revealed a rich knowledge base intimately connected to the seasonal and elevational progression of *Rhododendron* flowering (Hart and Salick 2017). Many species were differentiated by local names, and their uses included for fire wood, food, fodder, medicines for both people and livestock, and as a nectar source for honeybees. They were used in rituals, were an important part of seasonal festivals, and were an indicator in the ecological calendar. A similar attention to rhododendron seasonal and elevational progressions was evidenced in eastern Nepal (Ranjitkar 2012) and elsewhere in the eastern Himalaya, where rhododendrons serve diverse uses, including a beverage. Around Mt. Yulong, this knowledge came with broad consensus on the sequential progression of *Rhododendron* flowerings, and throughout the interviews, the connections of this progression to the seasons emerged as a crosscutting theme. For example, the flowering of the "buckwheat rhododendron," called *shouxma mgepqy* in the local Yi language, traditionally signaled to Yi informants the correct time to plant buckwheat (*Fagopyrum tataricum* or *F. esculentum*). However, indicators like these were reported to be now losing their value to the community, as the majority of interviewees reported confusing changes to the phenological sequence, with the flowering time of species now unpredictable in any given year.
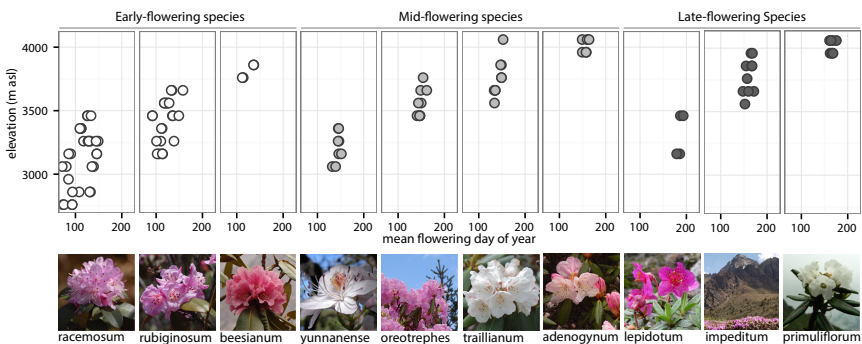
Figure 3. At Mt. Yulong, flowering times of rhododendrons in a given season are dispersed over elevation, and species within an elevational band flower in sequential progression. Each pane shows the elevation and mean flowering day of populations of one *Rhododendron* species. Shades indicate seasonal cohorts based on species mean flowering time: early-season (before day 130), mid-season (between day 130 and 160), and late-season (after day 160).

However, despite this attention to phenology, local people did not report observing a long-term advance in *Rhododendron* flowering time. To some extent, this was a surprise—if rhododendrons showed such clear responses to warming in our short-term ecological study, why then did local people not observe a change across the past several decades of warming temperatures? It could be that, historically, such close observations were never important, or that, with development, the local community had diverted towards different livelihoods and no longer pay close attention to such indicators. Such a transition has been observed in the Sagarmatha (Mt. Everest) and other popular tourism regions of Nepal, where extreme differences in livelihoods are evident between intensively visited trekking areas and the more remote areas just a few kilometers away. In Nepal, this division of livelihoods corresponds with a clear difference in perceptions of seasonal indicators and their use to time agricultural activities. A similar knowledge gradient is clear in the Mt. Yulong area between rural and urban areas (Hart and Salick 2017).

In this case of Yulong rhododendrons, the local observation—of no long-term advance—was borne out by a separate source of longer-term data, the *Rhododendron* specimens preserved in herbaria.

Himalayan *Rhododendron* phenology: natural history collections

Although herbarium specimens were collected to delineate and define new species, they and their associated labels also constitute a wealth of data on *when* and *where* Himalayan rhododendrons have flowered over the past century. There has been a recent explosion in the application of data from herbarium specimens to looking at long term responses to drivers such as climate change. These have included aspects of plant spatial ranges (Loiselle *et al.* 2007, Hereford *et al.* 2017), morphology (Law and Salick 2005, Beauvais *et al.* 2017), medicinal value (Souza and Hawkins 2017), and phenology (Primack *et al.* 2004, Willis *et al.* 2017). Increased attention has also been brought to refining methods which recognize the non-random way in which herbarium specimens are collected and attempt to quantify and account for their biases (Meyer *et al.* 2016, Daru *et al.* 2018).

To gain a deeper historical context for how rhododendrons of Mt. Yulong have been responding to annual changes in temperature, we photographed more than 10,000 herbarium specimens of the *Rhododendron* species that occur on Mt. Yulong (Hart *et al.* 2014). We supplemented the data available on these specimens with information from collector field books, diaries, and maps, and were able to extract reliable dates of collection and elevations of flowering for 1147 specimens. Using these data, we were able to corroborate the lack of directional change in mean flowering time over the recent decades of rapid warming which had been reported in our interviews with local

people. We were also able to suggest a possible explanation for the observed short-term responses not resulting in long-term directional changes. Using our record and seasonal data on temperatures from the Global Historical Climate Network (Lawrimore *et al.* 2011) we showed that mean flowering time advances with annual warming (2.27 days earlier per 1°C of warming) but is delayed with fall warming (2.54 days later per 1°C warming). This may be due to the effects of especially warm falls delaying the chilling that is physiologically required before overwintering buds will break dormancy and begin their spring growth.

The model we derived from herbarium specimens, some dating back more than 100 years, took into account annual warming, fall warming, and elevation. It matched the observations of local people, and also fit with our ecological observations.

New opportunities for monitoring *Rhododendron* phenology

In this work on Himalayan rhododendrons, the authors have seen the advantages of leveraging information and data from diverse sources. Although we are not the first to employ the specimens of past plant hunters and the knowledge of local peoples to inform and evaluate ecological hypotheses, it is worth reiterating what a wealth of useful data these sources represent. In the spirit of this *Rhododendron* research initiative, we would also like to make the point that rhododendrons in horticultural contexts represent a similar opportunity for data collection across broad spatial gradients.

Attention has recently been drawn to the value of *botanical* gardens in monitoring projects such as those discussed here (Primack and Miller-Rushing 2009), and data from well-documented personal plantings should be equally valuable. In many cases, well-known species are planted across a wide variety of climates and microclimates, offering an excellent opportunity for comparisons to be made. In some cases, such as the subscription-based seed collections of some of the early plant hunters in the Himalaya, documented wild seed collections have subsequently been distributed to both botanical gardens and private collections around the world. This opens up the unique opportunity to study the phenological behavior of close genetic relatives both in their native populations and simultaneously in widely distributed gardens in diverse climatic situations.

One small example of the potential of this sort of comparison is for *R. yunnanense*, which in our monitoring on Mt. Yulong was the exception to many of the generic patterns we observed. *R. yunnanense* flowered later than predicted by the herbarium-derived model, later with warmer annual temperatures, and without a significant change in flowering time over elevation. Intriguingly, plantings of *R. yunnanense* at the Royal Botanic Gardens Edinburgh (RBGE) monitored in the years 2002–2009 also flowered later with warmer temperatures (Harper *et al.* 2010).

To more systematically leverage this type of opportunity, it might be important for a nascent rhododendron network to take steps such as:

> a) identifying which gardens and living collections already have legacy (e.g., Wade 1979) or ongoing (as at RBGE) phenological observations that include rhododendrons;

> b) identifying species of interest and possibilities for linked monitoring of wild source-populations and garden plants;

> c) tracking specific seed collections and lineages with archival or population-genetic methods; and

> d) reviewing new tools recently promulgated to help with distributed data collection (e.g., Nature's Notebook www.usanpn.org/natures_notebook) and analysis (Hufkens *et al.* 2018) in wide-spread phenological monitoring projects.

This could be followed by designing or adopting a simple method for phenological monitoring. The monitoring method we mention above was shaped by the realities of transects across 1500+ elevational meters, but was also designed to be amenable both to quantitative analysis and to the methods used at the RBGE. A useful method in such a dispersed network would benefit from being easy to apply and record across diverse environments, while at the same time allowing comparison to data from existing legacy or ongoing data collection initiatives.

Ultimately, the benefits of such a network are not just in providing data to advance our understandings of plant responses to climate change, but also in recognizing what Anna Lawrence (2009) called "a more deeply integrated connection between observation and meaning." In this instance, it is a meaning that stems from the beauty of a *Rhododendron* coming into flower. This beauty is recognized alike by those who have lived with rhododendrons near their Himalayan villages, those who have collected and dispersed them, those who study them, and those who grow them.

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Rhododendron Natural Products as Sources of Novel Antibiotics

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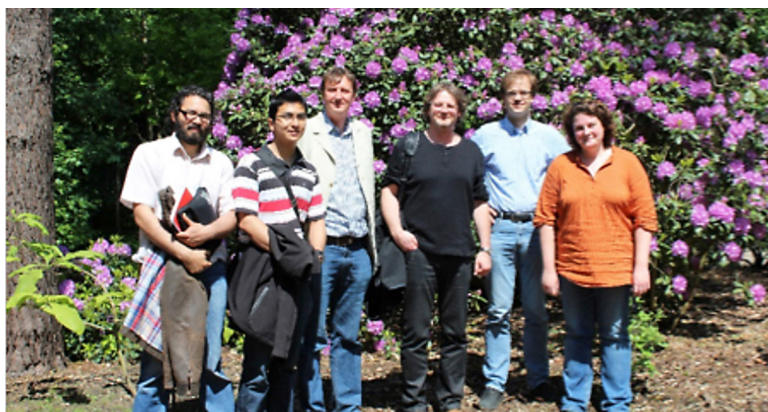
Researchers involved in Bremen *Rhododendron* project: From left to right, Ahmed Rezk, Abhi Shrestha, Nikolai Kuhnert, Matthias Ullrich, Dirk Albach and Jennifer Noelzen.

Introduction

Plants are admirable and creative chemists. Plants produce a myriad of natural products with fascinating and intricate chemical structures associated with fascinating biological activities. Plants have, over billions of years of evolution, created natural products as chemical solutions to their particular environmental challenges. The exact biological functions of most natural products is largely unknown to scientists, however, any compound produced by nature at a large energetic expense must be presumed to serve a purpose (Haslam 1986). As humans, we take advantage of these natural products in very different contexts. Most importantly, plants form the major part of our diet, containing nutrients, vitamins and further constituents maintaining and improving our health. Secondly, humans have exploited plants and extracts derived

from them, as sources of medicines. Traditional knowledge on the biological activity of plants has been transmitted and conserved over millennia by traditional healers and still helps today to relieve symptoms of disease or even cure diseases. Despite the triumph of allopathy, using synthetic drugs, over half of the global population still depends on medicinal plants as their primary source of healthcare. About half of all synthetic drugs are natural products themselves or derivatives of natural products, half of these of plant origin (Cragg *et al.* 2013, Saklani and Kutty 2008).

Consequently, investigating plant natural products constitutes a promising approach in discovering compounds with desirable biological activities, in particular medicinal properties. On our planet exist an estimated 400,000 different plant species (Firn and Jones 2003). Only a fraction of them has been investigated for their medicinal properties and an even smaller fraction investigated with respect to their chemical composition, leaving a lot of work to be done by scientists. *Rhododendron* forms no exception.

Why should, above all, *Rhododendron* be investigated as a potential source of new drugs? Firstly *Rhododendron* extracts are still used in a large number of ethno-medical applications for treatment of infections and cardiovascular diseases (Popescu and Kopp 2013). Secondly, *Rhododendron* species are found all over the world, except some regions in America and Africa, from temperate cold climates to tropical regions. Here, they needed to adapt and develop chemical solutions to a large diversity of environmental challenges. They are a diverse group of woody plants that belong to the family *Ericaceae* and are appreciated by the readers of this journal predominantly for their colorful flowers. Among the woody plants, *Rhododendron* is believed to be the most diverse genus with more than 1,200 species and countless cultivars described. Very few of them have been investigated for their biological activity and chemical composition (Jaiswal et al. 2012). Finally, the authors live in Bremen and Oldenburg, where the Rhododendron Park is a beloved local attraction, providing the world's second largest collection of *Rhododendron* plants. Hence, a fantastic source of study objects was available allowing to potentially valorize the plant collection while enjoying the beauty of the park.

In 2013, the Stiftung Deutscher Rhododendron Park decided on the initiative of its President the late Wolfgang Klunker, to use some of their funds to start a research project investigating the potential of *Rhododendron* as source of new antibiotic compounds. A multidisciplinary research team from Jacobs University Bremen and the University of Oldenburg, comprising expertise from phytochemistry, microbiology, botany, bioinformatics and cell biology started screening plant extracts for their antibacterial activity (Fig. 1). Some selected results of these investigations are summarized in the following sections, demonstrating clearly that *Rhododendron* does not only appeal and impress by the beauty of their flowers, but as well by their rich chemistry and promise for new medicines.
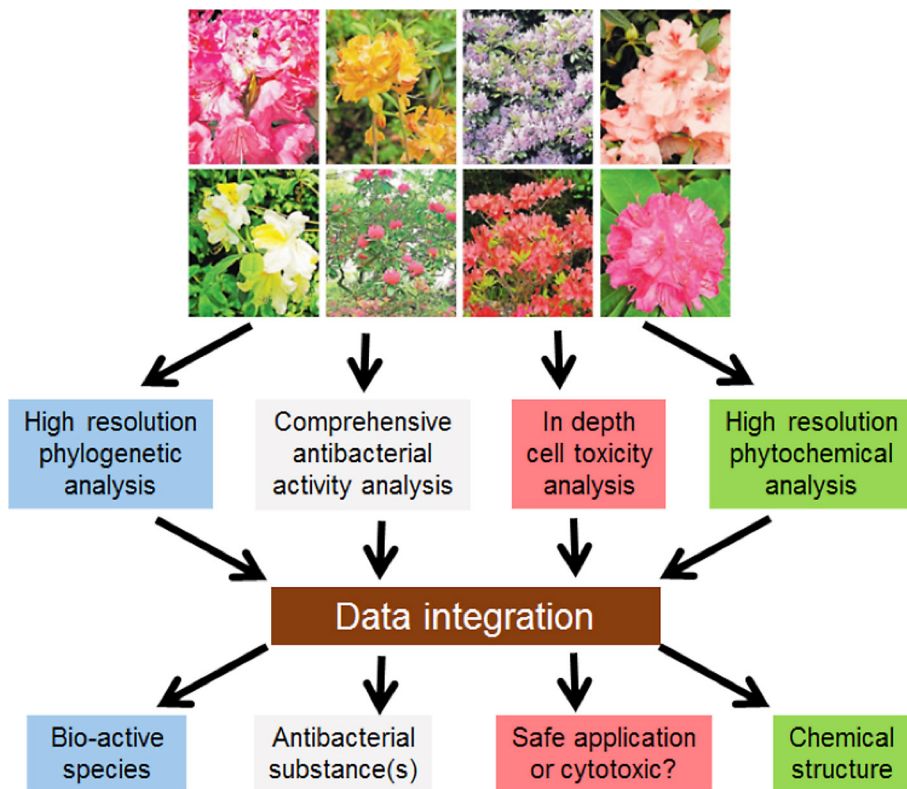
Figure 1: Basic approach of the Bremen *Rhododendron* project.

Basic chemistry of *Rhododendron*

Rhododendron plants have been investigated on rare occasions, identifying some of their natural products. The most famous compound in *Rhododendron* is grayanotoxin (Fig. 2.1, Li *et al.* 2015, Chen *et al.* 2004). This toxic diterpene is mainly found in the flowers of several *Rhododendron* species and, after collection by bees, accumulating in honey. Following consumption of grayanotoxin-rich honey, an intoxication associated with perspiration, dizziness and vomiting, also referred to as mad honey disease, results. In antiquity, both Greek and Roman armies under Pompeius Magnus suffered heavy defeats following unintentional mad honey consumption.

The color of *Rhododendron* flowers is caused by anthocyanins (Fig. 2.2, 2.3, Du *et al*. 2018). This particular class of compounds shows structural diversity and changes color upon change of flower tissue pH, co-pigmentation or metal complexation. Hence, a single class of compounds is responsible for the wide range of beautiful *Rhododendron*

flower color, in combination with adjustment of the pH value of the flower petal tissue. Many other flowers and fruit peels apply the same chemical principle.

Rhododendrons are furthermore rich in polyphenols. These compounds are generally known for their health benefits and popularized as antioxidants (Crozier *et al.* 2009). Polyphenols in *Rhododendron* are predominantly hydroxycinnamates (Fig. 2. Structure 4, Shrestha *et al.* 2017a) and proanthocyanidines (Fig. 2. Structure 6, Jaiswal *et al.* 2012) with structures that are equally encountered in many other edible plants such as red wine, dates, cranberry, cocoa, apples, coffee or tomatoes. Representative chemical structures of some natural products from *Rhododendron* are mentioned in Fig. 2.

Phylogeny of *Rhododendron*

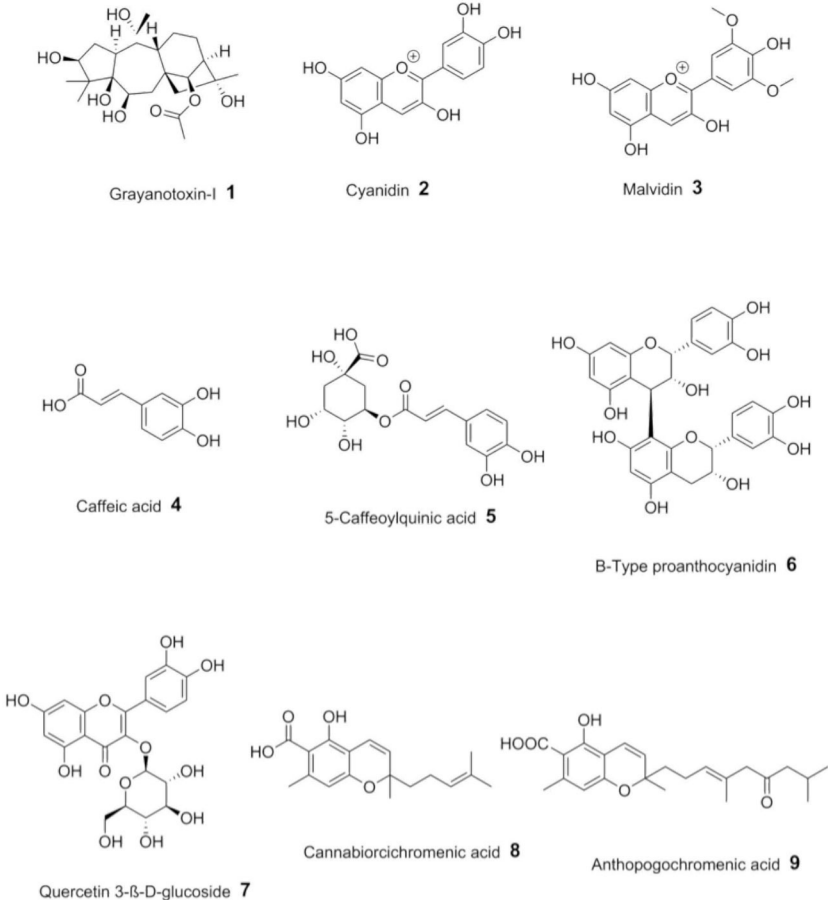Parallel to our search for novel antibiotics, we carried out detailed phylogenetic

Figure 2: Representative chemical structures of selected natural products from *Rhododendron*.

analyses of *Rhododendron* species, using a broad selection of DNA markers. This work supported recent reclassifications of *Rhododendron* on the subgenus and section level but also demonstrated large differences between plastid and nuclear DNA markers (Grimbs *et al.* 2017), which highlights the need for future phylogenomic analyses to resolve reliably the phylogenetic history of the genus.

With our updated phylogenetic tree in hand, we could address a series of basic scientific questions such as whether there is a correlation between phylogeny, biological activity and phytochemical profile. Antibacterial activity for example was predominantly observed in the subgenus *Rhododendron*. This finding reflects the bias of the chosen bacteria for screening, rather than the absence of antibacterial compounds in other subgenera. As another interesting finding, we observed that the ploidy level, which is the number of chromosome sets of a plant, has no influence on the number of natural products produced despite the fact that polyploidy predominates in groups rich in bioactive substances. Hence, an increased number of alleles does not necessarily lead to a richer and more diverse chemistry.

Chemotaxonomy of *Rhododendron*

In general, a typical *Rhododendron* plant produces between 50 and 300 different natural products. Most of them are common to all *Rhododendron* species and found widely in the plant kingdom. Some compounds, however, are unique to a certain subgenus or even plant species or hybrid. Identifying such compounds unique to a subgenus or even species is termed chemotaxonomy. With phylogenetic data and phytochemical profiles determined by liquid chromatography coupled to mass spectrometry (LC-MS), we could identify a series of such marker compounds. In particular, compounds from the class of hydroxycinnamates, a subgroup of polyphenols, were shown to be useful in this aspect (Shrestha *et al.* 2017a). Additionally, we investigated variations in chemical composition depending on the plant organs looking at leaves of different ages compared to flowers and fruits (Shrestha *et al.* 2017b). For few other plants, information of this type was available.

Antibiotics in *Rhododendron*

Why did we decide to look for new antibiotics in *Rhododendron*? Firstly, new antibiotics are desperately needed. A century ago, one third of the human population died from bacterial infections. This number has now dropped to below one percent, elevating modern antibiotics as medicinal chemistry's greatest triumph for humanity. However, many bacteria have ever since developed resistance to commercial antibiotics, necessitating novel active structures with new mechanisms of action (Payne et al. 2007). At the same time, most big pharmaceutical companies have left the field of antibacterial

research, for a combination of diverse factors including economic, scientific and political reasons (Silver 2011). Only two big pharmaceutical companies i.e., GSK and Sanofi are still actively pursuing antibacterial research. In the last two decades, only a single new antibiotic drug was launched to the market. Therefore, there is an urgent need to identify new promising structures.

In plants, many natural products aim at combatting bacterial pests. Plants are sessile organisms that cannot run away, so they need to find chemical solutions to fight off bacterial infections. Probably most plant natural products have evolved as chemical warfare agents against microorganisms and herbivores (Haslam 1986, Firn and Jones 2000, Firn 2004). The genus *Rhododendron* is exposed to multiple and diverse microbial environments growing in cold climate as well as in tropical regions on most continents. Thus, interesting structures must be expected possessing antibacterial properties.

Hence, we decided to screen around 200 different *Rhododendron* extracts for their antibacterial activity. This process involves several steps. Firstly, plant extracts are produced, which are tested in so-called agar plate diffusion assays, in which an antibacterial effect is established on a single selected bacterial strain. Around 20 *Rhododendron* extracts screened displayed the desired high antibacterial effect. For those minimal inhibitory concentrations (MICs) were determined to quantify their activity. Once identifying an antibacterial effect, we screened this extract for activity against a selection of further bacterial species to assess its effectiveness against other infectious agents (Rezk *et al.* 2015a).

A further 30 plant extracts displayed a lower antibacterial effect, most likely caused by a high concentration of polyphenol natural products. Polyphenols act in all plants as non-specific antibacterial agents against a broad spectrum of pest organisms. For medicinal use, they are unsuitable due to the high concentration required and their metabolic instability. They are typically metabolized by the gut microbiota. Many plants such as tea, coffee or quince utilize extremely high polyphenol concentrations in leaves or fruits, at times in the millimolar range, a million times higher than required for a drug, to combat pests.

In a second step, the toxicity for humans of the extract is assessed. For any antibiotic to be discovered, the compound in question must be toxic to bacteria, however at the same dose non-toxic to humans. Toxicity is very common in nature. Of the 400,000 plants on our planet an estimated 20,000- 40,000 are edible. All others are unpalatable or even toxic. Indeed, nature is the master of toxic compounds. Among the 50 most toxic compounds known to science, all are natural, not even one is of human synthetic origin. Consequently, as natural product scientists we are horrified by the common concept that natural or organic equates in popular culture to benign and healthy, a

bizarre if not preposterous concept advocated by irresponsible eco-ideologists.

Once a non-toxic extract with antibacterial activity is identified, in our work few extracts fell into this category (Figure 3; Rezk *et al.* 2015b), a process termed activity guided fractionation follows. Here, the extracts containing 50-300 detectable compounds are fractionated and retested. Active extracts are always kept and further purified until, at the very end, a pure chemical compound with the desired activity results. A combination of spectroscopic methods is then used to determine the chemical structure of the active compound, which could one day turn into a new antibiotic drug.

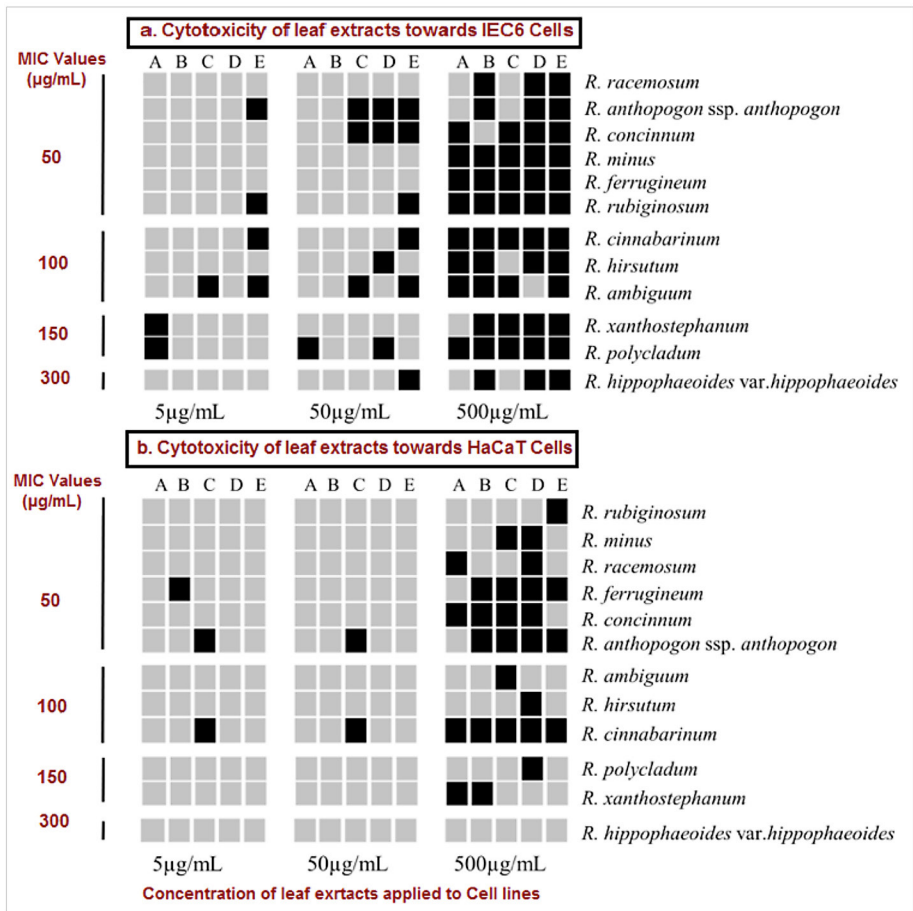The highlight molecule we identified from *Rhododendron collettianum*, a species

Figure 3: Summary of antibacterial testing and cytotoxicity assays cytotoxicity towards HaCaT cells and IEC-6 cells of *Rhododendron* extracts at different concentrations. Black boxes show an effect, grey boxes show no effect (Rezk *et al.* 2015b).

native to Afghanistan, is cannabiorcichromenic acid (CCA; Fig. 2. Structure 8, Said *et al.* 2017). This particular molecule not only displayed high antibacterial activity, it was as well non-toxic at antibacterial concentrations. From a structural point of view, it belongs to the class of the cannabinoid natural products, related in structure to the famous THC (tetrahydrocannabinol) found in cannabis. Since its publication, the compound has attracted interest from several sources, including producers of animal feeds requiring new antibiotics due to new legislation or the cosmetic industry looking for new antibacterial agents in deodorants. We will need to wait until further funding allows further development of this promising compound.

Bioinformatic analysis of data

Through a combination of phytochemical profiles with antimicrobial susceptibility and cytotoxicity, complemented by phylogenetic analyses (Figure 4), we combined all our collected data to identify seven potentially antimicrobial active but non-cytotoxic compounds (Grimbs *et al.* 2017). Exemplary bioactivity-guided fractionation for a promising *Rhododendron* species experimentally supports in fact one of these candidate lead compounds. By combining categorical correlation analysis with Boolean operations, we have been able to investigate the origin of bioactive effects in further detail. Intriguingly, we discovered clear indications of systems effects (synergistic interactions and functional redundancies of compounds) in the manifestation of antimicrobial activities in this plant genus (Grimbs *et al.* 2017). Additionally, we discovered that statistical analysis of data allowed the prediction of antibacterial compounds prior to actual experimental verification (Said *et al.* 2017). This aspect of work might facilitate drug discovery using natural sources in the future.

Conclusion

In summary, *Rhododendron* is exciting, not only due to gorgeous flowers but as well as a creative chemist. We could identify in excess of 300 different natural products in more than 100 *Rhododendron* extracts analyzed.
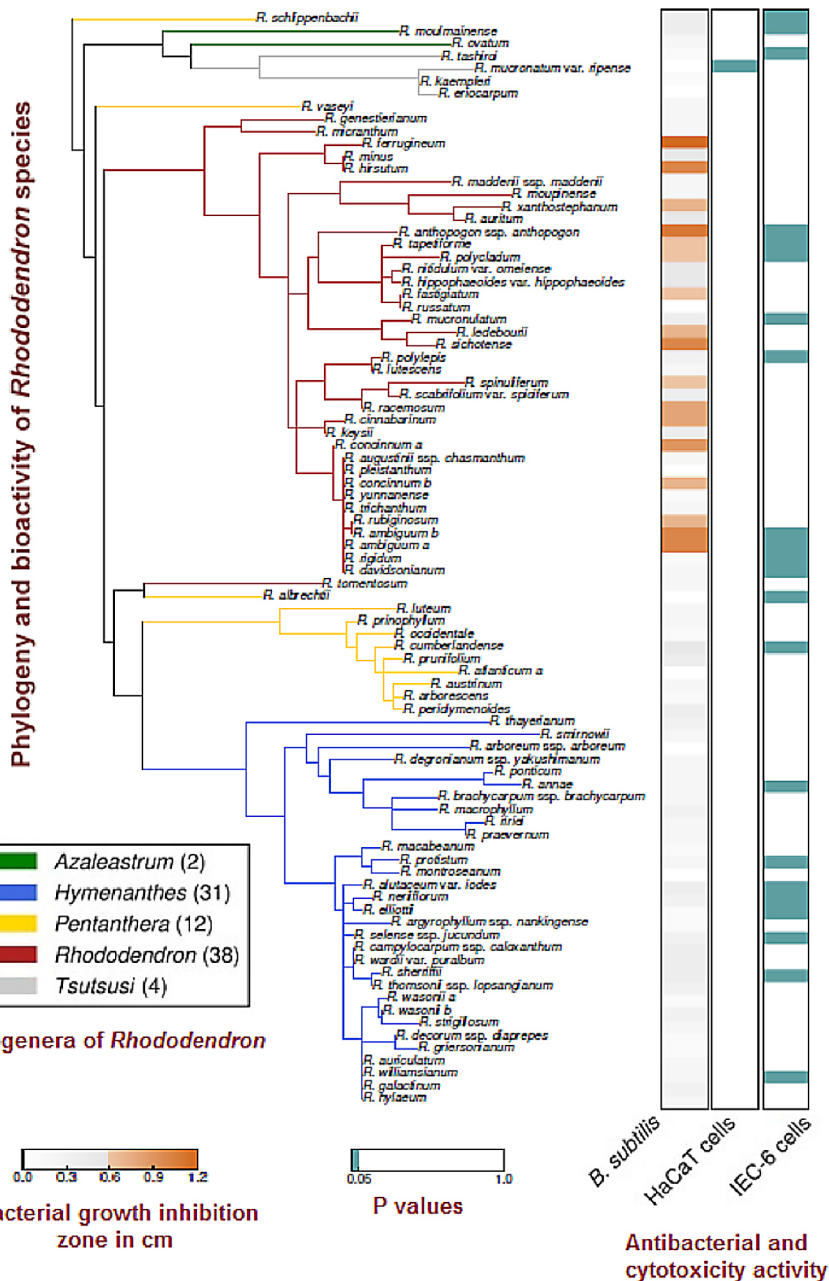
Figure 4: Phylogeny and bioactivities of *Rhododendron*. The phylogenetic tree of the 87 *Rhododendron* species is based on three genetic markers, *trn*K, *trn*L-F, and ITS. The three panels show the antimicrobial activity against *B. subtilis* (left), and the cytotoxicity towards HaCaT cells (middle) and IEC-6 cells (right) with respect to the given threshold and significance level, respectively (Grimbs *et al.* 2017).

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