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# Integration and macroevolutionary patterns in the pollination biology of conifers

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**Integration influences patterns of trait evolution, but the relationship between these patterns and the degree of trait integration is not well understood. To explore this further, we study a specialized pollination mechanism in conifers whose traits are linked through function but not development. This mechanism depends on interactions among three characters: pollen that is buoyant, ovules that face downward at pollination, and the production of a liquid droplet that buoyant grains float through to enter the ovule. We use a well-sampled phylogeny of conifers to test correlated evolution among these characters and specific sequences of character change. Using likelihood models of character evolution, we find that pollen morphology and ovule characters evolve in a concerted manner, where the flotation mechanism breaks down irreversibly following changes in orientation or drop production. The breakdown of this functional constraint, which may be facilitated by the lack of developmental integration among the constituent traits, is associated with increased trait variation and more diverse pollination strategies. Although this functional "release" increases diversity in some ways, the irreversible way in which the flotation mechanism is lost may eventually result in its complete disappearance from seed plant reproductive biology.**

**KEY WORDS:** Character correlation, integration, pollination biology, saccate pollen.

Many traits in animals and plants are integrated through shared function, shared development, or both (Olson and Miller 1958; Berg 1960; Murren 2002; Pigliucci and Preston 2004), and this should have pronounced effects on evolutionary patterns (Armbruster et al. 1999; Pigliucci 2003; Young and Badyaev 2006; Goswami et al. 2014). The evolutionary consequences of trait integration have most often been studied in systems such as the mammalian cranium (Cheverud 1982; Zelditch et al. 1992; Goswami 2006; Marroig et al. 2009; Goswami and Polly 2010) and the vertebrate jaw (Zelditch et al. 2008; Zelditch et al. 2009), where developmental and functional integration can result in evolutionary patterns as divergent as long-term stasis in some cases (Wagner and Schenk 2000) or diversification along coordinated pathways of character change in others (Albertson et al. 2005; Goswami et al. 2014).

Of course, trait systems show wide variation in the nature of their interactions and their degree of integration, and this should also contribute to the types of evolutionary patterns they exhibit (see Wagner and Schwenk 2000; Schwenk and Wagner 2001). For instance, in contrast to many of the examples mentioned above, traits can be integrated in terms of their function but not their development. Intricate pollination mechanisms in flowering plants often depend on the precise morphology and spatial orientation of floral parts such as stamens and stigmas, but these structures retain their individuality in the sense that they are not fused or otherwise directly linked through development (see Leins and Erbar 1990, 1997, on the “brushing” and “pump” mechanisms in sunflowers and relatives). We might expect such groups to exhibit different evolutionary patterns than clades whose floral parts are fused into a single functional structure more akin to a mammalian cranium (see Endress 1990; Endress 2001, on the “gynostegium” in orchids and milkweeds), but the specific relationships between macroevolutionary patterns and trait integration are not yet fully understood (Young and Badyaev 2006; Goswami et al. 2014).

To explore these issues, we focus here on the evolution of pollination biology in conifers, a group of around 630 nonflowering seed plant species including pines, cedars, and their relatives (see Farjon 2010). We study a pollination mechanism based on the proposed functional interaction among several developmentally unrelated traits: pollen grains with inflated air bladders called sacci (Fig. 1A, B), ovules (unfertilized seeds) that are oriented downward at pollination, and the presence of a pollination drop, an aqueous secretion produced by the ovules. In species exhibiting these characters, the ovules and the pollination drops they generate face downwards with respect to gravity (see Fig. 1C, D) and this orientation causes pollen with sacci, which are buoyant in water, to float upward through the drop and inside the ovule (Fig. 1E). This process is thought to help concentrate saccate pollen for fertilization and to screen out nonbuoyant foreign pollen and spores (Tomlinson et al. 1991; Tomlinson 1994; Runions and Owens 1996; Leslie 2010). In contrast, conifers with nonsaccate pollen generally lack downward-facing ovules, pollination drops, or both. These observations suggest that sacci function as part of an integrated pollination mechanism based on the physical flotation of pollen grains (Doyle, 1945; Tomlinson et al. 1991, Runions and Owens 1996; Salter et al. 2002; Leslie 2010), but this has not been tested in a detailed phylogenetic framework (see Tomlinson 2012).

In this study, we combine ancestral character state reconstructions and maximum likelihood (ML) transition rate models with a well-sampled phylogeny of conifers (Leslie et al. 2012) to test how potential functional integration among these characters influences their evolutionary patterns. We first test whether the presence of saccate pollen grains is actually correlated with the production of pollination drops and downward-facing ovules in a phylogenetic context. We then test different hypotheses of how this character suite has changed over time; specifically, whether sacci are lost following changes in drop production or ovule orientation that would disrupt the operation of a flotation-based pollination mechanism (i.e., Tomlinson et al. 1991; Tomlinson 1994). Our results suggest that these traits are functionally linked: their evolution is correlated and changes in pollination biology are associated with apparently irreversible losses of the flotation mechanism and saccate pollen. We further suggest that the lack of developmental integration among these traits may facilitate this process, where new combinations of character states can readily evolve from a system that has strong functional interactions but which is nonetheless somewhat “loose” in its total level of integration.

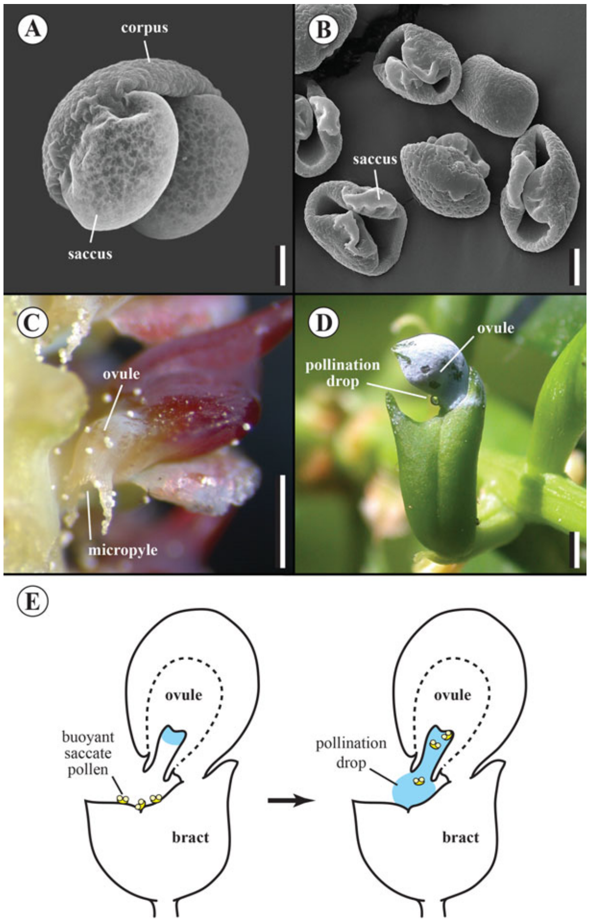

**Figure 1.** Saccate pollen morphology and the pollen flotation mechanism in living conifers. (A) SEM micrograph of saccate pollen of black pine (*Pinus nigra*) showing large sacci and the central pollen body (corpus). (B) SEM micrograph pollen of Huon pine (*Lagarostrobus franklinii*) showing poorly developed “vestigial” sacci. (C) Ovule of *Pinus mugo* covered in pollen grains and with two arms surrounding the downward-facing micropyle, or ovule opening. Although a pollination drop is not visible in this specimen, it is secreted between the arms and pollen grains that encounter it float upwards inside the ovule. (D) Seed cone of *Podocarpus neriifolius* at pollination showing a single downward-facing ovule at an early stage in the production of a small aqueous pollination drop. (E) Schematic longitudinal section through the seed cone of *Podocarpus neriifolius* after full production of the pollination drop, when the buoyant saccate pollen floats upwards into the micropyle. In this particular species, the ovule is embedded in modified cone scale tissue that is in turn borne on a bract, a modified leaf-like structure. Scale bars = 10 μm in A, B; 0.5 mm in C; 2 mm in D.

## Methods

We used a previously published large-scale phylogeny of conifers (Leslie et al. 2012) and limited our data set to species sampled in that study. In total, our data comprise information from 466 living taxa (ca. 75% of extant species), which includes 88% of extant conifer genera. Of the species not included in the phylogeny, almost all (ca. 90%) come from clades in which pollination mechanisms have been otherwise well characterized (e.g., *Pinus*, *Abies*, *Podocarpus*, Cupressaceae); our data therefore are representative of pollination strategies across conifers as a whole.

For all species in the phylogeny, we compiled information relating to pollen morphology (presence of sacci, buoyancy, and pollen wall structure) and pollination mechanism (drop production, ovule orientation, and seed cone orientation at pollination) from literature sources, supplemented with personal observations (see Table S1). We treat the presence of sacci and pollen buoyancy as two separate characters because some species have pollen grains with poorly developed sacci (Fig. 1B). Such grains are not buoyant in water, but the presence of "vestigial" sacci provides potentially important information about the sequence of character state changes involved in the loss of sacci. In *Dacrydium araucarioides* and *Lagarostrobus franklinii*, both species with "vestigial" sacci for which pollen buoyancy was not previously known, we observed the behavior of fresh pollen in water droplets.

Pollen morphology is known for almost all conifer species, as is the orientation of ovules and seed cones at pollination. The presence or absence of a pollination drop has been investigated and described in representative species of most conifer genera and supra-generic groups (comprising ca. 20% of the species in our data). Species for which the presence or absence of a pollination drop has not been directly investigated (for example, many members of species-rich clades such as *Juniperus*, *Pinus,* and *Podocarpus* have not been specifically studied) were scored the same as better-known, closely related species because they otherwise share features of cone structure and ovule orientation (see Table S1). In a small number of cases (ca. 5% of the species in the phylogeny), we were unable to find adequate information to reliably score pollination biology (especially the production and orientation of pollination drops) and these taxa were not included in our study (see Table S1). Species may exhibit some variation in the size and shape of their sacci, as well as the exact angle of cone and ovule orientation, but, in general, these traits are similar within species.

Using the phylogeny and data on pollen morphology, ovule orientation and cone orientation, we reconstructed ancestral states with both parsimony and ML. We then tallied the inferred number of losses and gains of both sacci and pollen buoyancy. We also recorded whether groups with non-buoyant pollen (i.e., those that lack sacci or have only "vestigial" sacci) showed a change in ovule orientation and/or the presence of pollination drops compared to sister taxa with buoyant saccate pollen. Parsimony analyses were performed using Mesquite 2.75 Build 566 (Maddison and Maddison 2011) and ML analyses were performed using the rayDISC function in the R package *corHMM* (Beaulieu et al. 2013).

We then tested whether character states were correlated using an extension (see Beaulieu and Donoghue 2013) of existing correlated character transition models (see Pagel 1994). We assigned each species a combined character state consisting of three binary traits: the presence or absence of sacci, the presence or absence of buoyant pollen, and the presence or absence of downwards-facing pollination drops (a combination of pollination drops with downwards-facing ovules). Since all nonsaccate conifer grains are nonbuoyant, the two character state combinations that implied buoyancy for nonsaccate grains were eliminated and transitions to and from these states were not allowed.

We tested for correlations among characters by asking whether models of transition rates among combined character states (character-dependent models) provided a better fit to the data than models that fit each trait separately (character-independent models). The likelihood of a correlated (dependent) character model is proportional to the probability of observing the distribution of combined character states given a single model of evolution that defines transitions among all possible states. The likelihood of an uncorrelated (independent) character model is the product of the separate probabilities of observing the data given the transition rates applied to each individual trait (see Pagel 1994). We fit two types of correlated and uncorrelated models to the data, one in which all transition rates were equal (ER; equal rates) and one in which all rates could be different (ARD; all rates different).

To test the likely sequence of character state changes, we fit five different ARD combined character state models that tested different hypotheses regarding how saccate pollen is lost or gained in relation to ovule orientation (see Fig. 2). In all models only one state change is allowed at each transition, and as noted above, two possible combined character states were not allowed because they are not physically possible in conifers (i.e., nonsaccate pollen that is also buoyant). Model fit and parameter estimates were obtained using the corDISC function in the R package *corHMM* (Beaulieu et al. 2013), and model support was assessed using the Akaike information criterion (AIC; Akaike 1974).

The first model ($ARD_1$) is a null that imposes no constraints on the fourteen possible state transitions. In the second model ($ARD_2$), buoyancy can only be lost following loss of the downward-facing drop (caused by a shift in ovule orientation or drop production) and cannot be regained. Similarly, sacci can only be lost following the loss of buoyancy and they cannot be regained. In this model, therefore, the full flotation suite cannot be reassembled once its individual components have been lost. The third model ($ARD_3$) is similar to $ARD_2$, except that

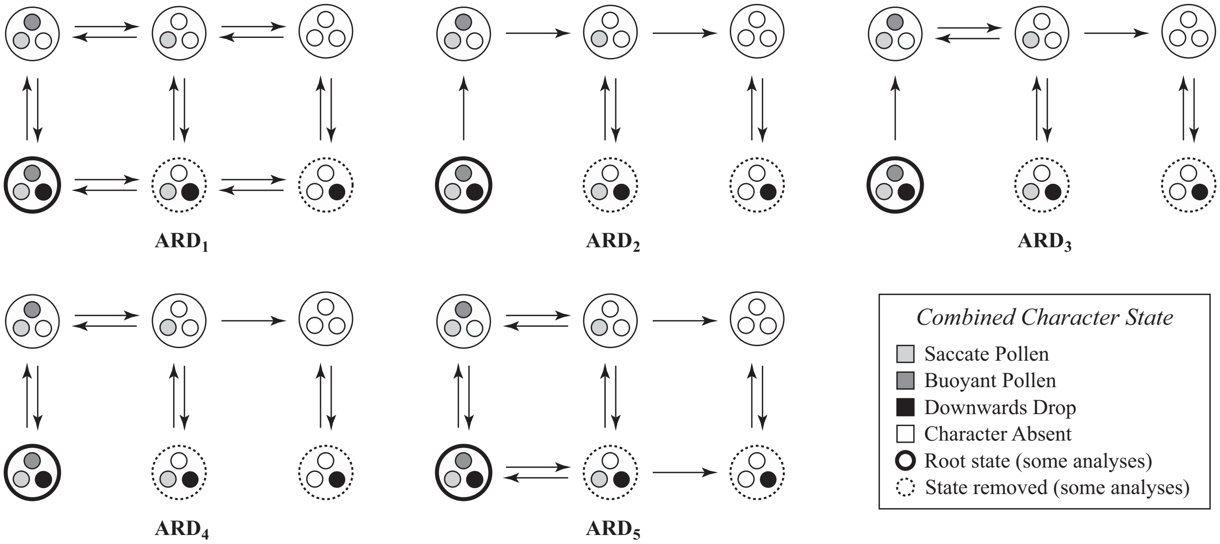

**Figure 2.** Graphical depiction of the five "all rates different" (ARD) models of correlated character evolution tested in this study, where ovule orientation and the presence of pollination drops have been combined into a single character state ("downwards drop"). Arrows indicate allowed transitions between states. ARD$_1$ is a null model in which all possible transitions can occur, and ARD$_2$ – ARD$_5$ represent different hypotheses of the order in which characters change (see main text for a full discussion). We also tested alternative versions of each model where the root state was fixed to have the fully functioning flotation mechanism (heavy circle). For some analyses of the Pinaceae and Podocarpaceae clades, additional character combinations were removed because they do not occur among living taxa (dashed circles).

buoyancy (but not sacci) can be regained. In the fourth model (ARD$_4$), all three components of the full flotation suite can be regained after they have been lost. In the fifth model (ARD$_5$), both sacci and buoyancy can be lost without a prior change in ovule orientation, but sacci cannot be regained once lost. We also tested a variant of each of these models (denoted as ARD$_1$*–ARD$_5$*) where the root character state was fixed to be saccate, buoyant, and with downward-facing drops.

In addition to fitting these models to the complete conifer data set, we also separately analyzed the major subclades corresponding to the conifer families Cupressaceae *sensu lato*, Pinaceae, Podocarpaceae, and Taxaceae (characters in the Araucariaceae are invariant). In Podocarpaceae and Pinaceae, the correlated ARD models were modified to eliminate transitions to and from one additional combined character state that is not found in any extant member of these clades (see Fig. 2; the nonsaccate, nonbuoyant, downward-facing drop state). In Pinaceae, we also removed an additional character state from the models (vestigial sacci, nonbuoyant, with downward-facing drops). Again, this state is not found in extant Pinaceae, and models that did not include it received higher AIC support than models that did (see Table S2).

# Results

Nine conifer groups (clades or individual species) lack buoyant saccate pollen in our data (Fig. 3; Table 1), which together include 217 species (47% of the species sampled). Seven of the nine clades or individual species with nonbuoyant pollen also lack downward-facing pollination drops, either because their ovules are consistently lateral or erect at pollination, because they do not produce drops, or both (Fig. 3; Table 1). One group with non-buoyant pollen (the *Sciadopitys* + Taxaceae + Cupressaceae clade) has variable drop orientation and one additional species (*Lagarostrobus franklinii;* clade 5 in Fig. 3) with nonbuoyant pollen has consistently downward-facing drops.

Six of the groups with nonbuoyant pollen grains show shifts in either drop production or ovule orientation relative to saccate sister clades (Table 1), regardless of whether the ancestral states of these characters were reconstructed using parsimony or ML. Likelihood reconstructions suggest that all nine nonbuoyant groups represent losses from an ancestrally buoyant, saccate condition (Table 1; with two separate losses of buoyancy in *Tsuga*), although parsimony reconstructions are equivocal regarding the ancestral state for five of the nine groups (Table 1; see also Fig. S1). In no cases, however, are sacci unambiguously shown to reevolve from nonsaccate ancestors. Likewise, there are no unambiguous cases where the complete flotation-based pollination mechanism itself is known to reevolve once lost.

Transition rate models of character evolution provide further support for the correlated evolution of sacci and ovule characters, as well as for the irreversible loss of sacci and the flotation-based mechanism. When all conifer species are analyzed together, correlated models of character evolution among sacci, buoyancy, and drop orientation are strongly favored based on AIC scores (Table 2). The single best-supported model (Table 2; ARD$_3$*)

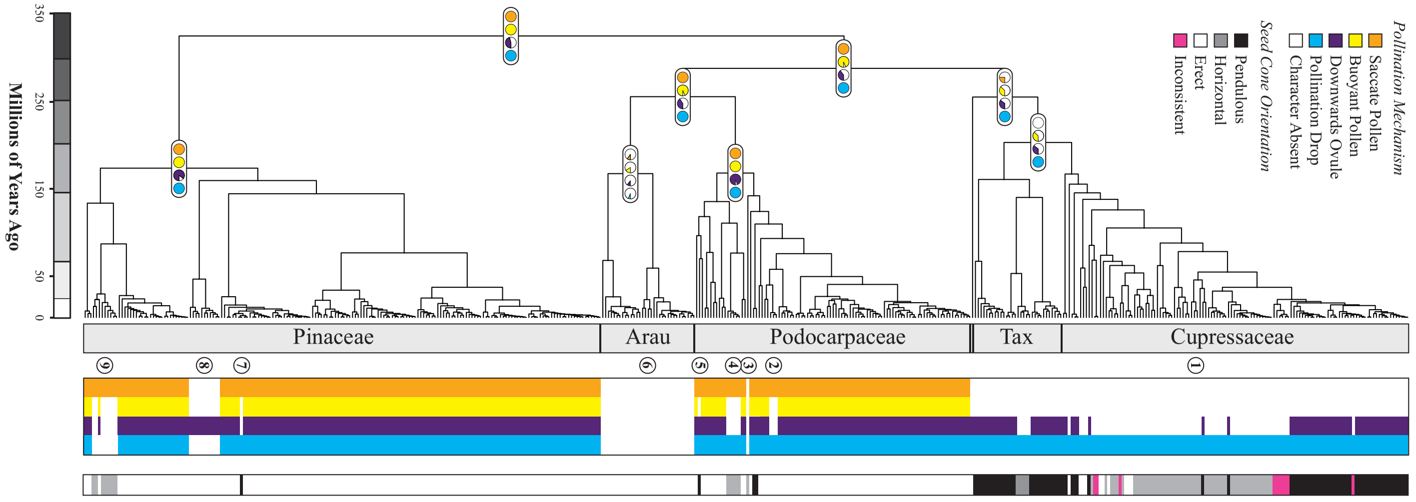

**Figure 3.** Phylogenetic distribution of characters relating to pollination mechanism and seed cone orientation among extant conifers. Ancestral character states in backbone nodes were determined by maximum likelihood reconstruction methods and are shown by colored pie graphs within the ellipses, where the percentage of color represents the likelihood of the state. Numbers on the phylogeny represent clades or individual species that do not have buoyant pollen, and correspond to the numbers listed in Table 1.

**Table 1.** Features of conifer groups (either clades or single species) that lack buoyant saccate pollen.

| Conifer groups lacking buoyant saccate pollen | Drop faces down? | Change in ovule characters relative to sister clades? [ML/MP] | Reconstructed as loss of buoyancy? [ML/MP] |
|---|---|---|---|
| (1) *Sciadopitys* + Tax.+ Cup. | Variable | ?/No | Yes/? |
| (2) New Caledonian *Dacrydium* | No | Yes/Yes | Yes/Yes |
| (3) *Saxegothaea conspicua* | No | Yes/Yes | Yes/? |
| (4) *Phyllocladus* | No | Yes/Yes | Yes/Yes |
| (5) *Lagarostrobus franklinii* | Yes | No/No | Yes/Yes |
| (6) Araucariaceae | No | Yes/Yes | Yes/? |
| (7) *Picea orientalis* | No | Yes/Yes | Yes/Yes |
| (8) *Larix* + *Pseudotsuga* | No | Yes/Yes | Yes/? |
| (9) *Tsuga* (except *T. mertensiana*) | No | ?/? | Yes/? |

The number for each group corresponds to those shown on the phylogeny in Figure 3A. "Drop" in the first column refers to the aqueous pollination drop secreted by the ovules. "Ovule characters" in the second column refers strictly to the orientation of the ovules relative to gravity and presence/absence of a pollination drop. The third column refers to whether the lack of pollen buoyancy in each group is reconstructed as a loss from an ancestral buoyant condition. The sister lineage to *Tsuga* (*Nothotsuga*) was not sampled in our data set, any change in ovular characters relative to sister taxa is therefore not known. New Caledonian *Dacrydium* includes *D. araucarioides*, *D. lycopodioides*, and *D. guillauminii*. A "?" indicates an ambiguous or equivocal reconstruction. ML, maximum likelihood; MP, maximum parsimony; Tax, Taxaceae; Cup, Cupressaceae.

also has a fixed root state corresponding to an operating flotation mechanism (saccate, buoyant, and with downwards-facing drops), although it is worth noting that parameter estimates were similar in an equivalent version without a fixed root. This model is one in which buoyancy, and ultimately sacci, are lost in response to the loss of downwards-facing pollination drops (either due to the loss of the drop itself or from a change in ovule orientation). The model estimates a low transition rate (0.002) away from the operational flotation-mechanism (Fig. 4A; lower left-hand state) followed by a very high transition rate to the state consisting of "vestigial" sacci without a functioning flotation mechanism (Fig. 4A; upper middle state). From there, the model suggests that descendant clades may occasionally re-evolve a downward-facing drop (Fig. 4A; lower middle state) or fully transition to a nonsaccate state (Fig. 4A; right-hand states) where drop orientation may vary among descendant taxa.

Transition rate models also suggest that neither the pollen flotation mechanism nor sacci are ever regained once they are lost; rates for these transitions were either not allowed in the best-fit model or were estimated as zero. There is a single instance among extant conifers (the hemlock species *Tsuga mertensiana*) that suggests buoyancy may have been regained after its loss (other hemlock species do not have buoyant pollen), and this is responsible for the relatively high rates estimated for this transition (5.89 in Fig. 4A). Although this species has been described as having functional sacci (Owens et al. 1998), the physical properties of *T. mertensiana* pollen need to be investigated further. Alternatively scoring this species as non-buoyant does not substantially change our results. In this case, the best-supported model for conifers becomes an $ARD_2$* model, in which neither the flotation mechanism, pollen buoyancy, nor sacci are regained once lost (see Supporting Materials for a more detailed discussion of *Tsuga*).

Although models of correlated character evolution are strongly supported for conifers as a whole, this is not the case when major subclades of conifers are analyzed separately. Clades that are reconstructed as ancestrally nonsaccate (Cupressaceae *sensu lato* and Taxaceae) are best fit by uncorrelated models of character evolution, while clades that are reconstructed as ancestrally saccate (Pinaceae and Podocarpaceae) are best fit by correlated models (Table 2). This distinction is evident in the structure of the correlated models as well, because they suggest that once either buoyancy or sacci are lost, ovule orientation can vary freely in descendant nonsaccate species or clades (Fig. 4A).

The saccate clades differ in which correlated models are favored. The best-fit model in Podocarpaceae is a modified $ARD_2$* where, as mentioned previously, an additional combined character state has been removed because it does not occur among extant species. This model reconstructs the loss of the flotation mechanism, pollen buoyancy, and sacci as irreversible (Fig. 4B). In Pinaceae, the best-fit model is similar to conifers as a whole (Fig. 4C; an $ARD_3$*), where pollen buoyancy can be regained once lost but neither sacci nor the fully operational flotation mechanism can be regained.

## Discussion

Our analyses support the idea that a suite of seed cone, ovule, and pollen characters function in an intricate pollination mechanism that is ancestral to living conifers and has been maintained over hundreds of millions of years. This is consistent with the fossil record, where even some of the earliest conifers possessed this suite of characters (Mapes 1987; Clement-Westerhoff, 1988; Leslie et al. 2008). One potential advantage of this mechanism, and a possible reason for its long-term persistence, is that it increases both the number and proportion of conspecific grains available for fertilization (Tomlinson et al. 1991; Runions and Owens 1996; Leslie 2010). This may increase male gametophyte competition and/or promote greater female choice, as pollen vigor and siring success vary across individual conifers (Nakamura and Wheeler 1992; Aronen et al. 2002).

Despite these potential advantages, the flotation mechanism has been disassembled and lost numerous times over conifer evolution, with no noticeable impact on broad patterns of evolutionary "success"; that is, extant nonsaccate clades do not have lower net diversification rates (Table S3) nor is the breakdown of the mechanism associated with shifts in net diversification rate identified in previous studies of conifers (Fig. S3; see also Leslie et al. 2012; Leslie et al. 2013). While some methods used to identify rate shifts have been questioned (Rabosky and Goldberg 2015), visual inspection of the tree alone (Fig. 3) suggests that it is highly unlikely that the loss of saccate pollen is associated with any changes in conifer diversification. Nevertheless, the breakdown of the flotation mechanism does have important consequences for trait evolution, and it provides an interesting example of how functional interactions may shape broader patterns of evolution.

### FUNCTIONAL INTEGRATION AND THE EVOLUTION OF CONIFER POLLINATION BIOLOGY

Pollen morphology and ovule orientation appear to be strongly linked in conifers, which explains the correlated patterns of character change such as the loss of buoyant pollen following shifts in ovule orientation. In other ways, however, the characters that compose the flotation mechanism are not highly integrated. For example, pollen morphology and ovule orientation are not developmentally related: whether a pollen grain produces sacci does not influence the successful initiation, growth, and positioning of an ovule prior to pollination. Furthermore, even the functional interaction among the characters may not always be as strong as it would appear. At least one saccate *Pinus* species is able to set seed if ovules have been manually repositioned from their pre-

**Table 2.** Support for models of character evolution in all conifers and selected conifer subclades, with the best-supported models in bold.

| | AIC Values | | | | |
|---|---|---|---|---|---|
| Model | All Conifers | Cupressaceae | Taxaceae | Podocarpaceae | Pinaceae |
| Uncorrelated ER | 243.38 | **54.85** | **11.00** | 63.38 | 82.24 |
| Uncorrelated ARD | 240.24 | 56.22 | 11.66 | 69.13 | 85.92 |
| Correlated ER | 248.86 | 73.99 | 16.30 | 61.57 | 80.56 |
| Correlated $\text{ARD}_1$ | 183.72 | 79.85 | 35.62 | 53.74 | 57.09 |
| Correlated $\text{ARD}_1$* | 183.13 | — | — | 52.32 | 55.27 |
| Correlated $\text{ARD}_2$ | 184.33 | 65.86 | 21.47 | 46.39 | 58.41 |
| Correlated $\text{ARD}_2$* | 180.75 | — | — | **43.17** | 55.64 |
| Correlated $\text{ARD}_3$ | 176.45 | 67.86 | 23.47 | 48.39 | 50.04 |
| Correlated $\text{ARD}_3$* | **172.87** | — | — | 45.17 | **47.27** |
| Correlated $\text{ARD}_4$ | 175.44 | 69.86 | 25.47 | 49.16 | 50.63 |
| Correlated $\text{ARD}_4$* | 174.87 | — | — | 47.17 | 49.27 |
| Correlated $\text{ARD}_5$ | 180.48 | 75.85 | 31.47 | 52.27 | 55.09 |
| Correlated $\text{ARD}_5$* | 180.78 | — | — | 50.32 | 53.27 |

An asterisk indicates a model variation where the root state has been fixed as saccate, buoyant, and with downwards-facing drops. This variation does not apply to the Cupressaceae or Taxaceae clades, which lack saccate pollen. In Pinaceae and Podocarpaceae, correlated ARD models are modifications that eliminate transitions to and from additional character states (see main text for more information). ER, equal rates; ARD, all rates different.

ferred downward orientation (McWilliam 1958), although it is not known how general this phenomenon is. But it is perhaps not surprising that the flotation mechanism has broken down a number of times, if its constituents form a somewhat "loose" constellation of traits in the sense that they are not developmentally integrated and there are intermediate arrangements of the system that can still function (for the alternative pattern of labile traits coming together to form an integrated system, see Ogburn and Edwards 2009).

Assessing exactly how likely this system is to breakdown is somewhat complicated, however, because the selective pressures that cause the loss of pollen flotation are not clear. The distribution of traits in living species suggests the order in which characters changes have occurred (e.g., taxa such as *Picea orientalis* and *Phyllocladus* where ovule orientation has shifted relative to sister clades but whose pollen still possesses "vestigial" sacci;Tomlinson et al. 1997; Runions et al. 1999), but shows few patterns otherwise. For example, taxa that have lost the flotation mechanism are broadly similar to other species in their pollination syndrome (all conifers are wind pollinated) and life history strategies, and they do not obviously share geographic or climatic features that would suggest an environmental driver for the breakdown of the flotation mechanism (although this needs to be investigated further). Living clades do show, however, a wide diversity of alternative pollination strategies relating to ovule position and pollen grain germination, which in some cases may be even more effective than flotation (see Colangeli and Owens 1989; Owens et al. 1998). So in a broader sense, the "loose" integration among reproductive traits in conifers may favor not only the breakdown of the ancestral flotation mechanism but also the evolution of diverse alternative pollination strategies, because it allows a variety of clade-specific intermediate states to exist and remain generally functional.

Regardless of the exact reasons why the flotation mechanism breaks down, it is a good example of how decoupling traits from a functional constraint may "release" them in an evolutionary sense, resulting in higher levels of variation in descendent clades. For example, seed cones in the nonsaccate Cupressaceae clade are oriented in a wide variety of angles (Fig. 3; see Dörken and Jagel 2013), presumably to minimize interference between air currents carrying pollen and cone-bearing branches, whose exact architecture can be highly variable in this clade. This kind of pattern is a general feature of integrated trait systems (see Wagner and Schwenk 2000; Schwenk and Wagner 2001), but may be particularly prevalent in systems where constituent traits are not directly linked through development and may therefore be more likely to evolve independently. In the flowering plant clade Malpighiaceae, for example, flower orientation increased in Old World lineages after the loss of an oil-bee pollination mechanism that maintains just one flower orientation in ancestral New World taxa (Davis et al. 2014). In systems with a high degree of developmental integration, on the other hand, major shifts or losses in function should result in more co-ordinated changes among constituent traits (see Goswami et al. 2014) or their wholesale loss, as in the loss of an eye or a limb and its components (see Jeffery 2001; Wiens and Slingluff 2001; Yamamoto et al. 2004; Skinner et al. 2008).

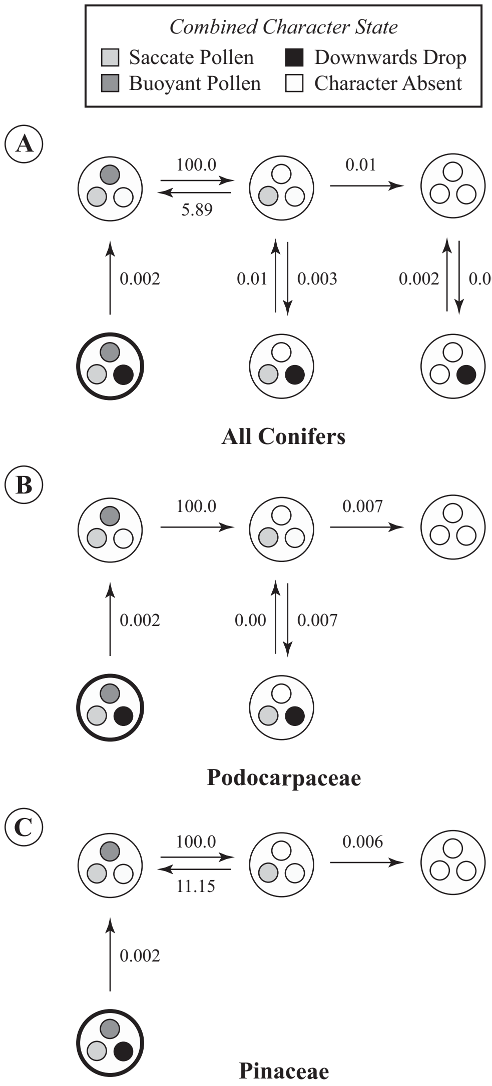

**Figure 4.** Best fit models of character evolution for (A) all conifers, (B) Podocarpaceae, and (C) Pinaceae with rate estimates given for each allowed transition. Models and trait combinations follow those depicted in Figure 2. The specific best-fit models, as determined by AIC score, were an $ARD_3$, a modified $ARD_2$, and a modified $ARD_3$ model, respectively. In Podocarpaceae and Pinaceae, character states that are not observed in living taxa were removed; these variations received higher support than models that included them. In all cases, the best-fit models were ones where the root state was fixed to have the fully functioning flotation mechanism (state with heavy circle).

## MACROEVOLUTIONARY CONSEQUENCES OF POLLEN FLOTATION LOSS

Although the breakdown of the flotation mechanism is associated with increased diversity in pollination strategies and seed cone orientations, it limits reproductive biology in at least one important way: there are no clear cases in which either sacci or the flotation-based mechanism has ever reevolved after it has been lost. These results are also consistent with the fossil record; the Araucariaceae, Cupressaceae, and Taxaceae each have at least a 200 million year history and there is no evidence for the reevolution of saccate grains in these clades (see Harris, 1979; Stockey 1994; Taylor et al. 2009). The breakdown of pollen flotation, and the subsequent loss of sacci, therefore appears to permanently eliminate the use of this mechanism in descendant clades.

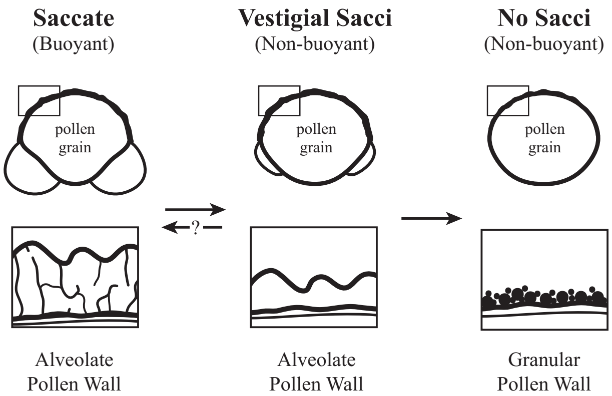

**Figure 5.** Schematic representation of conifer pollen grains and their wall structure. The alveolate pollen wall of saccate grains is spongy with a complex network of internal partitions and compartments, and sacci represent regions of the grains where these compartments are expanded. Pollen grains with vestigial sacci often have simpler wall structure, but still possess some compartments within the pollen wall and are here still considered alveolate. Nonsaccate grains have an irregular, granular pollen wall that lacks these compartments. Arrows indicate possible evolutionary transitions based on best-fit character models. The question mark indicates that the transition back to buoyant saccate from vestigial sacci depends on an ambiguous trait reconstruction within the hemlock (*Tsuga*) clade.

The structure of the pollen grains themselves may be the key to understanding this directionality. Species with saccate pollen have a structurally complex pollen wall with irregular internal partitions and compartments termed alveolae, while species with nonsaccate pollen have a simpler, less organized granular pollen wall that lacks them (Fig. 5; see Kurmann, 1990; Kurmann and Zavada 1994). Sacci are localized, inflated compartments of the alveolate wall that form by internal expansion of the wall during development (Dickinson and Bell 1970; Rowley et al. 2000). Once wall development has been simplified, it may be that the processes responsible for the formation and expansion of sacci are deleted from the pollen developmental repertoire, or that it is simply no longer possible for them to have a structural effect without the presence of alveolae.

Whatever its underlying mechanism, the irreversible loss of sacci will gradually remove pollen flotation from seed plant reproductive biology, if only because its loss is a one way street and its presence is neutral with respect to diversification (at least among living conifers). We might then expect seed plants to show a type of passive macroevolutionary trend, one resulting from diffusion in a bounded evolutionary space (McShea 1994) rather than from long-term directional selection (e.g., Vermeij 1993; Hunt and Roy 2006; Finkel et al. 2007; Gorzelak et al. 2012). In this particular system, however, evolutionary space is constrained by the ratcheted, unidirectional loss of a key functional trait rather than an upper or lower bound, as may be the case in animal body size evolution (Stanley 1973; but see Alroy 1998). The result is an aggregate evolutionary trajectory formed by the breakdown of pollen flotation in numerous individual clades for a variety of specific reasons.

Such an interpretation is broadly consistent with the fossil record. Although there are only two living lineages of saccate seed plants (Pinaceae and Podocarpaceae), sacci were much more widespread among major Paleozoic and Mesozoic seed plant groups, including the Callistophytales, Caytoniales, Corystospermales, Glossopteridales, some Peltaspermales, and early conifers of the Paleozoic (see Clement-Westerhoff 1987; Leslie 2008; Taylor et al. 2009). However, more work is needed on the phylogenetic relationships of extinct seed plants (see Hilton and Bateman 2006; Doyle 2008) and the evolutionary history of sacci in particular to determine if the decline of pollen flotation is due more to its gradual loss within lineages, the wholesale extinction of saccate gymnosperm groups, of a combination of both.

## Conclusions

Pollen morphology, ovule orientation, and the presence of pollination drops evolve in a correlated manner in conifers, which suggests that these traits are functionally integrated in a pollination mechanism based on the flotation of pollen grains. This mechanism has repeatedly broken down over conifer evolution, however, which may have been facilitated by the relatively "loose" degree of integration among the constituent traits. That is, these traits are not developmentally linked and they appear to retain some level of individual function without the operation of the flotation mechanism. The breakdown of the flotation mechanism appears to "release" traits such as ovule and seed cone orientation, which show greater variability in descendent clades, and is also associated with the evolution of a more diverse array of specific pollen capture mechanisms in conifers. At the same time, the evidently irreversible breakdown of the flotation mechanism appears to impose a long-term macroevolutionary trend in seed plant reproductive evolution, resulting in the gradual elimination of flotation and saccate pollen.

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### DATA ARCHIVING

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1.** Character data used in this study.
**Table S2.** Support for transition rate models of character evolution in Pinaceae that allow transitions to a state with vestigial sacci and downwards-facing drops (referred to as "with state") and those that exclude them ("without state").
**Table S3.** Results of BiSSE analysis.
**Figure S1.** Ancestral character states in backbone nodes as determined by parsimony reconstruction methods, where half-filled circles indicate ambiguous character states.
**Figure S2.** Support for transition rate models of character evolution if Tsuga mertensiana pollen is scored as non-buoyant in A) all conifers and B) Pinaceae.
**Figure S3.** Ancestral character state reconstruction for saccate pollen using BiSSE.