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RESEARCH ARTICLE

Toxicity of the spiny thick-foot *Pachypodium*

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PREMISE OF THE STUDY: *Pachypodium* (Apocynaceae) is a genus of iconic stem-succulent and poisonous plants endemic to Madagascar and southern Africa. We tested hypotheses about the mode of action and macroevolution of toxicity in this group. We further hypothesized that while monarch butterflies are highly resistant to cardenolide toxins (a type of cardiac glycoside) from American *Asclepias*, they may be negatively affected by *Pachypodium* defenses, which evolved independently.

METHODS: We grew 16 of 21 known *Pachypodium* spp. and quantified putative cardenolides by HPLC and also by inhibition of animal Na^+/K^+-ATPase (the physiological target of cardiac glycosides) using an in vitro assay. *Pachypodium* extracts were tested against monarch caterpillars in a feeding bioassay. We also tested four *Asclepias* spp. and five *Pachypodium* spp. extracts, contrasting inhibition of the cardenolide-sensitive porcine Na^+/K^+-ATPase to the monarch's resistant form.

KEY RESULTS: We found evidence for low cardenolides by HPLC, but substantial toxicity when extracts were assayed on Na^+/K^+-ATPases. Toxicity showed phylogenetic signal, and taller species showed greater toxicity (this was marginal after phylogenetic correction). Application of *Pachypodium* extracts to milkweed leaves reduced monarch growth, and this was predicted by inhibition of the sensitive Na^+/K^+-ATPase in phylogenetic analyses. *Asclepias* extracts were 100-fold less potent against the monarch compared to the porcine Na^+/K^+-ATPase, but this difference was absent for *Pachypodium* extracts.

CONCLUSIONS: *Pachypodium* contains potent toxicity capable of inhibiting sensitive and cardenolide-adapted Na^+/K^+-ATPases. Given the monarch's sensitivity to *Pachypodium*, we suggest that these plants contain novel cardiac glycosides or other compounds that facilitate toxicity by binding to Na^+/K^+-ATPases.

KEY WORDS Apocynaceae; cardenolide; cardiac glycoside; chemical ecology; *Danaus plexippus*; Madagascar; monarch butterfly; Na^+/K^+-ATPase; plant–insect interactions; sodium-potassium pump; southern Africa; stem-succulent milkweeds.

Pachypodium is a genus of 21 spiny and stem-succulent species in the Apocynaceae, with a center of diversity in Madagascar (Burge et al., 2013). *Pachypodium* diversified in open and arid habitats, including five species in southern Africa, and have growth forms ranging from dwarf shrubs that spread along the soil surface to large bottle trees >10 m in height. Although *Pachypodium* spp. have long been of interest to horticulturalists, relatively little scientific study has been conducted on these iconic plants beyond their systematics and habitat associations (Lee, 1912; Lavranos and Röösli, 1996, 1999; Rapanarivo et al., 1999; Rowley, 1999; Luthy, 2004; El-Kashef et al., 2015). We were interested in the toxicity of *Pachypodium* spp., due to suggestions in the literature that they possess potent chemistry and their use on poison arrows by native African societies (Helly, 1905; Meyer et al., 1964; Hoffman and Bigger, 1980; Neuwinger, 1998; El-Kashef et al., 2014). Given the geographically isolated diversification of this genus, we were particularly interested in evolutionary drivers of toxicity and the potential for novel chemical defenses to emerge that may have diverged from the classic coevolutionary compounds of the Apocynaceae (Malcolm, 1991; Agrawal et al., 2012). Indeed, such divergences of isolated taxa are often theorized to be drivers of novel defenses (Renwick, 2002; Cappuccino and Arnason, 2006; Davis et al., 2015).

Given the iconic nature of *Pachypodium* spp., the availability of seed for growth in a common environment (Fig. 1), and

FIGURE 1. Representatives of our greenhouse-grown *Pachypodium* species near the time of leaf harvest: (A) *P. rutenbergianum*, (B) *P. decaryi*, (C) *P. saundersii*, and (D) *P. geayi*. Photos by Ellen Woods.

a recent phylogenetic analysis of the genus (Fig. 2; Burge et al., 2013), we first sought to characterize their defensive chemistry and its mode of action. Plants in the Apocynaceae are widely known for their toxicity, with various genera containing alkaloids and cardenolides, the latter being a type of cardiac glycoside that inhibits the animal sodium-potassium ATPase (Na^+/K^+-ATPase; Agrawal et al., 2012). Thus, we aimed to compare their potency in relation to North American *Asclepias* and to test hypotheses about the impact of *Pachypodium* defenses on animal species adapted and not adapted to the Apocynaceae. Early research on *Pachypodium* was equivocal about the presence of alkaloids (Abisch and Reichstein, 1960), and our preliminary analyses using Dragendorff reagent were negative for alkaloids. However, early work suggested the possibility of cardiac glycosides or related compounds with properties similar to *Digitalis* (Helly, 1905; Meyer et al., 1964). Our preliminary observations were positive for Na^+/K^+-ATPase inhibition and HPLC peaks with absorbance measured at 220 nm, and thus we focus here on cardiac glycosides.

Cardiac glycosides are remarkable steroidal toxins, known in ~25% of the Apocynaceae, and are dominated by cardenolides in this plant family (Malcolm, 1991; Agrawal et al., 2012). Cardenolides together with bufodienolides make up the cardiac glycosides, which function by binding to Na^+/K^+-ATPase. The sodium-potassium pump is a critical cellular enzyme occurring in all animal cells and is responsible for several primary functions, including the maintenance of cell resting potential. Recent work has found novel compounds in the Apocynaceae (e.g., trisaccharides), not traditionally included as cardiac glycosides, that nonetheless gain their toxicity from binding to Na^+/K^+-ATPase (Rajashekar and Shivanandappa, 2017). Despite the ubiquitous target site of cardiac glycosides in animal cells, hundreds of adapted insect species specialize on cardenolide-containing plants (Petschenka et al., 2013b; Bramer et al., 2015; Agrawal, 2017; Petschenka et al., 2017). Indeed, in the case of the North American milkweeds (*Asclepias* spp.), several orders of insects have convergently evolved a small number of genetic substitutions in their Na^+/K^+-ATPase, allowing them to tolerate cardenolides (by lowering binding affinity) and exploit these toxic plants (Dobler et al., 2012). For example, the monarch butterfly, *Danaus plexippus*, has a highly resistant Na^+/K^+-ATPase (Vaughan and Jungreis, 1977), which is critical for its sequestration of cardenolides for its own defense against predatory birds (Petschenka and Agrawal, 2015). For highly specialized herbivores such as the monarch, it is unclear the extent to which they can tolerate novel defensive chemistry that has evolutionarily diverged in relatives of their host plants.

In the present study, we asked the following specific questions: (1) Do *Pachypodium* spp. contain cardiac glycosides measurable by liquid chromatography and application to a sensitive Na^+/K^+-ATPase in vitro? (2) What is the distribution of sodium pump toxicity in *Pachypodium* in relation to phylogeny, geography, and growth form? (3) Are monarch butterfly caterpillars, which are highly adapted to cardenolides, affected by *Pachypodium* extracts in feeding trials? And (4) what is the relative potency of *Pachypodium* vs. *Asclepias* extracts when tested on a sensitive Na^+/K^+-ATPase and that of the highly adapted monarch butterfly? In particular, we predicted that because *Pachypodium* defenses evolved independently from monarch butterflies, they may exhibit relatively stronger potency against the cardenolide-adapted monarch than the cardenolides produced by North American milkweeds. The extent to which such chemically mediated interactions are specific vs. generalized within taxa (i.e., a botanical family) is unclear, and therefore this study provides a window into how species diversification is associated with defense–offense interactions.

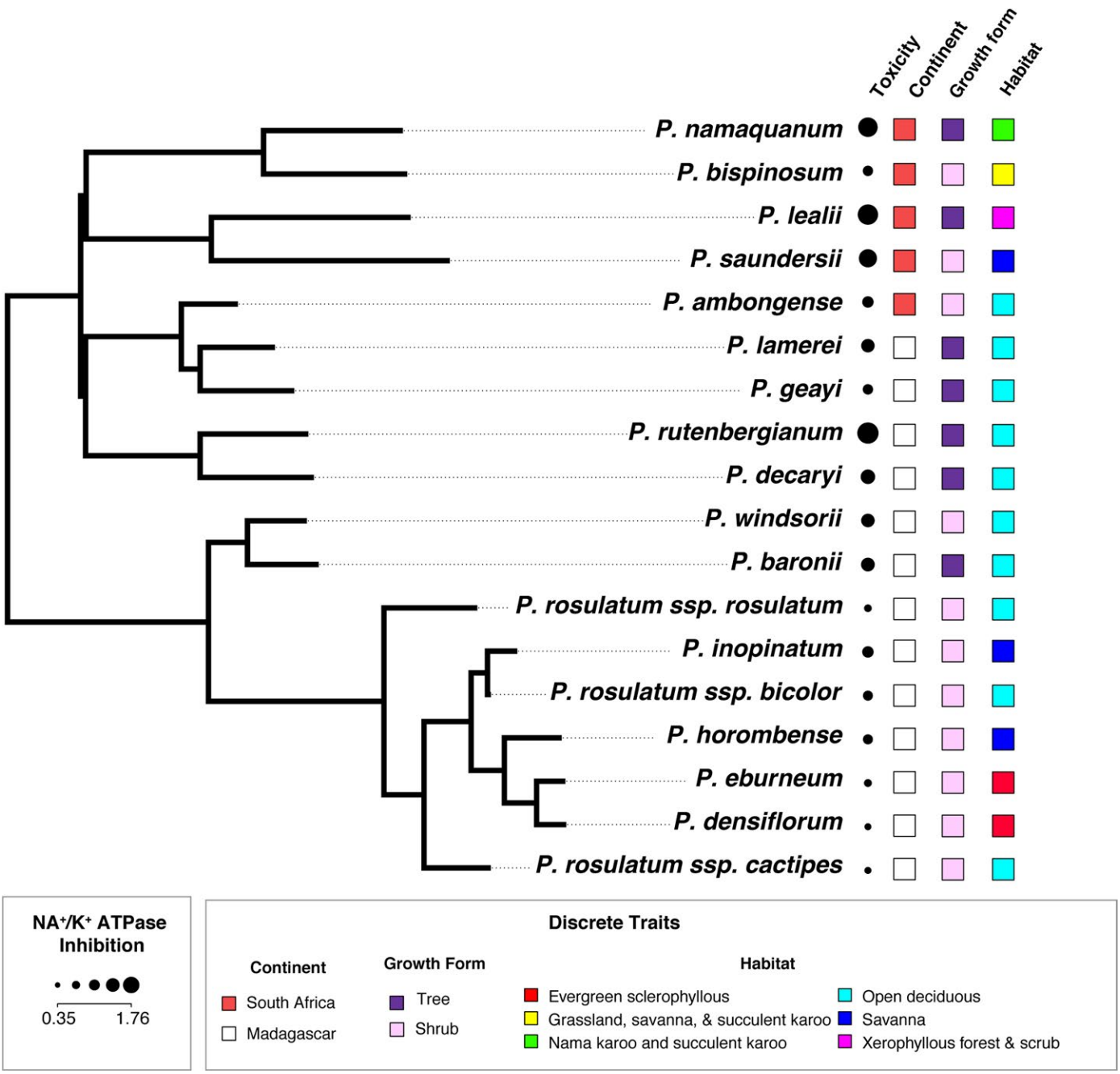

FIGURE 2. Consensus phylogeny of *Pachypodium*. A comprehensive phylogeny was pruned to 16 of the 21 known *Pachypodium* species and three of the six subspecies of *P. rosulatum*. Also indicated are the toxicity (Na^+/K^+ inhibition), geographic origin, growth form, and habitat association of each species.

MATERIALS AND METHODS

Plant growth and species identification

Eighteen *Pachypodium* taxa including three subspecies of *P. rosulatum*, were grown from seed (Fig. 1 and Appendix 1), germinated on moist paper towels in Petri dishes, and grown in clay pots containing a 4:4:1:1 ratio of potting soil, sand, vermiculite, and perlite. *Pachypodium lealii*, *P. namaquanum*, and *P. bispinosum* were obtained as small, growing plants. At least three replicate plants, typically from two sources, were maintained per species (Appendix 1). All plants were randomized in a common greenhouse and minimally watered and fertilized. Leaf tissues were taken after 1 yr of plant growth. To verify the identity of species, we sequenced the ITS1-5.8S-ITS2 nuclear ribosomal region and the trnL-trnF chloroplast region and compared results to voucher specimens used for constructing the phylogeny of *Pachypodium* (Burge et al., 2013). Plants were also physically compared to literature descriptions of morphology to confirm species identifications (Rapanarivo et al., 1999; Luthy, 2004). Following these authors,

growth form was distinguished between shrubs and trees; the latter designation was given to species with maximum heights >3 m in natural populations.

Comparisons of *Pachypodium* spp. were made with foliar tissue from four *Asclepias* spp. chosen to span a range of toxicity: *A. verticillata*, *A. syriaca*, *A. curassavica*, and *A. linaria* (listed here in order from very low to among the highest concentration of cardenolides for *Asclepias*; Rasmann and Agrawal, 2011). *Asclepias* were grown in a growth chamber in potting soil. Leaves were harvested, freeze dried, ground, and stored at room temperature.

Extraction and HPLC analysis of cardenolides

Leaf samples were freeze dried and ground into fine powder on a mixer mill (Retsch, Haan, Germany) using 3 mm metal beads. We extracted 50 mg of leaf powder in 1 mL 100% methanol spiked with 20 µg digitoxin (Sigma Aldrich, St. Louis, Missouri, USA) and ~30 FastPrep beads (MP Biomedicals, Santa Ana, California, USA) in 2 mL plastic screw-cap vials (Sarstedt, Nürmbrecht, Germany). Samples were agitated twice for 45 s at a speed of 6.5 m/s on a FastPrep-24 homogenizer, followed by centrifugation at 14,000 rpm for 12 min. The supernatant was evaporated at 35°C on a vacuum concentrator (Labconco, Kansas City, Missouri, USA), resuspended in 200 µL 100% methanol and filtered through a 0.45 µm filter (Kinesis, Berlin Township, New Jersey, USA).

HPLC was conducted on extracts of the four *Asclepias* spp. noted above and five *Pachypodium* spp. representing a range of toxicity (*P. rutenbergianum*, *P. geayi*, *P. densiflorum*, *P. namaquanum*, and *P. rosulatum* ssp. *rosulatum*) (total $n = 96$). We analyzed 15 µL of each sample by HPLC using a Gemini C18 reversed phase column (3 µm, 150 × 4.6 mm; Phenomenex, Torrance, California, USA) and an Agilent 1100 instrument with diode array detection. Cardenolides were eluted at a constant flow of 0.7 mL/min with a gradient of acetonitrile and water as follows: 0–2 min at 16% acetonitrile; 2–25 min from 16% to 70%; 25–30 min from 70% to 95%; 30–35 min at 95%; followed by 10 min reconditioning at 16% acetonitrile. Peaks were recorded at 218 nm, and absorbance spectra were recorded at 200–300 nm. Peaks showing a characteristic single absorption maximum between 214 and 222 nm were considered cardenolides. Concentrations of cardenolide compounds were calculated by relating peak areas to the area of the internal digitoxin standard. Representative chromatograms and absorbance spectra are given in Appendix S1 (see Supplemental Data with this article).

Na^+/K^+-ATPase assay

We quantified the biological activity of cardiac glycosides from plant extracts following the methods of Petschenka et al. (2013b), using Na^+/K^+-ATPase from the porcine cerebral cortex (Sigma-Aldrich, MO, USA). Na^+/K^+-ATPase activity was measured as the amount of inorganic phosphate (Pi) enzymatically released from ATP in the presence of K^+ (Na^+/K^+-ATPase active) minus the amount of Pi released in the absence of K^+ (Na^+/K^+-ATPase inactive). Ten milligrams of freeze-dried ground tissue from each sample was weighed out and extracted using a FastPrep-24 homogenizer, as above, but without digitoxin standard. Dried supernatants were then resuspended in 250 µL 10% DMSO. Two additional dilutions were prepared from this working stock solution: 1:10 and 1:100, by volume, in 10% DMSO. All three dilutions were tested with the ATPase in order to produce an inhibition curve for each sample.

Reactions were performed in 96-well microplates on a BioShake iQ microplate shaker (Quantifoil Instruments, Jena, Germany) at 200 rpm and 37°C. Each reaction was carried out in a total volume of 100 µL and consisted of 20 µL sample, 100 mM NaCl, 20 mM KCl, 4 mM MgCl2, 50 mM imidazole, 0.0015 units porcine Na^+/K^+-ATPase (Sigma A7510), and 25 µL ATP (ditrissalt, pH adjusted to 6.5 with Tris, 2.5 mM final well concentration). All reagents were combined in a master mix on ice and added to the plant extract just prior to incubation. After 20 min, reactions were stopped by the addition of 100 µL of 10% SDS/0.05% antifoam A. Inorganic phosphate released from enzymatically hydrolyzed ATP was stained by adding 100 µL Taussky-Shor reagent and photometrically quantified by measuring absorbance 700 nm. To account for coloration of plant extracts, we included a background well for each reaction well with identical content but lacking KCl. On each microplate we also included wells with an uninhibited control, a completely inhibited reaction by 10-2 M ouabain, and a calibration curve made with ouabain ranging from 10-3 to 10-8 M.

Absorbance values of reactions were corrected by their respective backgrounds and calculated as percent residual activity using reference points of the uninhibited reaction as 100% and the completely inhibited reaction as 0% residual activity. Based on the residual enzymatic activity inhibited by a biological sample at three concentrations, we estimated the sigmoid dose-response curve using a logistic function with the upper and lower asymptotes fixed to 100% and 0% residual activity, respectively (function *gnls* in the *nlme* package for R). For each sample, we calculated the relative dilution at the inflection point (i.e., residual enzymatic activity of 50%, or IC50) and estimated the cardiac glycoside concentration of the undiluted samples in ouabain equivalents based on the calibration curve.

We conducted two sets of analyses of the porcine Na^+/K^+-ATPase. Leaves from four or five individual plants from each of the 18 taxa were assayed on the sodium pump, then each extract was run twice independently, and then all were averaged for analyses ($n = 81$ biological replicates). In addition, a second set was run on the Na^+/K^+-ATPase with samples from the four *Asclepias* spp. and five *Pachypodium* spp. run on the HPLC noted above (4 replicates; $n = 36$). We simultaneously ran these samples on the highly resistant monarch Na^+/K^+-ATPase. For the monarch sodium pump assays, reparations of the monarch Na^+/K^+-ATPase were made from brains of adult *D. plexippus* butterflies that had been frozen alive at −80°C and thawed on ice for dissection. For each Na^+/K^+-ATPase preparation, four individual butterfly brains with the thoracic ganglia were pooled and homogenized in 2 mL deionized water. Aliquots of these pools were freeze dried, stored (−80°C), and used for assays subsequently. For use in the enzymatic assays, monarch Na^+/K^+-ATPase preparations were thawed in cold deionized water, vortexed, sonicated, and centrifuged.

To test the difference in our estimates of cardenolide concentrations based on HPLC vs. the sensitive porcine ATPase, we used the following analysis of variance (ANOVA) model: plant genus, cardenolide estimation method, plant species(genus), and plant genus × estimate method. To test the relative sensitivity of the cardenolide-adapted monarch ATPase compared to the sensitive porcine ATPase, we compared inhibition of each enzyme by extracts from the five *Pachypodium* spp. and four *Asclepias* spp. using the following ANOVA model: plant genus, ATPase source, plant species(genus), and plant genus × ATPase source.

Monarch growth assays and in vitro inhibition of the sodium pump

To test the impacts of leaf extracts on the growth of herbivores, we devised a method in which monarch larvae (*D. plexippus*) were individually fed common milkweed (*A. syriaca*) leaves painted with extracts from each of the 18 *Pachypodium* taxa. This approach had three benefits. First, it allowed us to assay whole-animal toxicity of *Pachypodium* extracts in addition to specific sodium pump toxicity. Second, monarchs, which are highly adapted to milkweed toxins, don't naturally feed on *Pachypodium*, but our assay facilitated their feeding on the novel compounds. Third, our assay removed physical differences between *Pachypodium* spp., as they vary tremendously in leaf toughness, thickness, and pubescence.

An average of seven larvae and three individual *Pachypodium* plants were employed for each of the 18 species or subspecies (n = 135 leaf disk assays). Samples were prepared with 50 mg powder from freeze-dried leaves as described above, omitting the digitoxin standard, and extracted with 1.5 mL 95% EtOH instead of methanol. Extracts were then treated as described above, except that they were resuspended in 750 μL 95% EtOH and then stored at −80°C. Leaf disks were cut using a 1.5 cm core from young, fully expanded leaves of fresh, field-collected *A. syriaca*. Leaf disks were randomized and painted with 15 μL of extract on both the top and bottom sides. Controls were painted with 15 μL of 95% EtOH on both sides. Leaf disks were allowed to dry and were then suspended on a pin so that larvae were allowed to feed on both the top and bottom of the leaf in a Petri dish containing moist filter paper and sealed with Parafilm (see Appendix S2). Monarch eggs were from a laboratory colony and were incubated at 30°C. All larvae used in the bioassay hatched within the same day. Larvae were each placed on a leaf disk and allowed to feed for 5 d. Petri dishes were stacked and covered to eliminate light gradients. After 2 d, leaf material was replaced with newly prepared disks, and larvae were removed and weighed on day 5.

Phylogenetic analysis of *Pachypodium* cardiac glycosides

A Bayesian posterior distribution of phylogenetic trees for the combined nuclear ribosomal (ITS) and chloroplast (trnL-trnF) data of Burge et al. (2013) was obtained as in that study, with the exception that two runs were conducted, sampling 10,000 trees per run; 500 trees each were randomly chosen from the post-burn-in posterior distributions of each run and merged to create a set of 1000 trees for comparative analysis. The DNA alignment is provided in Appendix S3. Phylogenies were rooted using the outgroups used in Burge et al. (2013), a selection of species from genera closely related to *Pachypodium*. Trees were pruned to the 18 taxa studied here, retaining one branch-tip per species as follows. For taxa that were monophyletic in all 1000 unpruned trees (six total: *P. baronii*, *P. decaryi*, *P. horombense*, *P. rosulatum* ssp. *rosulatum*, *P. rosulatum* ssp. *gracilius*, and *P. windsorii*), an individual was chosen at random for inclusion in each of the 1000 pruned phylogenies. For three species that were non-monophyletic (*P. densifiorum*, *P. eburneum*, and *P. lamerei*), DNA barcodes obtained for the plants included in the study were used to inform pruning as follows. Newly obtained ITS sequences were BLASTed against a local database of the ITS sequences used to build the phylogeny. Resulting percent sequence identity was used to guide the pruning of the posterior distribution of trees for the character analyses by selecting an individual to include in the phylogeny with a probability proportional to its ITS sequence identity with the individual in the study. This resulted in a set of phylogenies with high representation of individuals that have high similarity to the ITS sequence of individuals in the experiments, but also incorporated some uncertainty. When possible, phylogenetic comparative analyses were run using the set of 1000 trees to account for topological uncertainty. However, for instances where a single tree was required (e.g., single-tree plots and figures), a maximum clade credibility tree with median node heights was calculated using TreeAnnotator in BEAST (Drummond et al., 2012).

We used several comparative approaches to investigate patterns of sodium pump inhibition across the *Pachypodium* phylogeny. First we tested the phylogenetic signal of sodium pump inhibition using Blomberg's K statistic with the "phylosignal" function in the "picante" package (Kembel et al., 2010). Next we calculated the phylogenetic independent contrasts between sodium pump inhibition and monarch growth in the extract bioassay using the "pic" function in the "ape" package (Paradis et al., 2004). We also tested whether patterns of sodium pump inhibition macroevolution supported scenarios in which variation in the abundance of sodium pump inhibition across species was explained by impacts of habitat, geography, or growth form.

Plant habitat affiliation and apparency have long been studied as predictors of plant defense in a comparative context (Agrawal, 2007). In particular, Malcom (1991) reviewed the evidence that more apparent species in Apocynaceae were better defended by cardiac glycosides than less apparent species. Indeed, there was some support for larger and longer-lived species containing higher cardiac glycosides. Here, we employ two measures of *Pachypodium*'s apparency, a qualitative designation of growth form (tree vs. shrub) in addition to a quantitative measure (maximum height) as predictors of plant defense in phylogenetic analyses.

We compared several evolutionary models of sodium-pump-inhibition phenotypic evolution across the *Pachypodium*. First, we fit a single-rate multivariate Brownian motion (BM) model, BM1. This model reflects a random-walk process by which the probability of sodium-pump-inhibition divergence increases uniformly through time regardless of habitat, continent, or growth form status. We then evaluated models where lineage geography, habitat, or growth form affected the sodium-pump-inhibition phenotype trajectory by comparing single-rate models to multi-rate scenarios. In particular, we fit three multi-optima OU model (OUM) with separate sodium pump inhibition optima (θ) for each state (but σ^2 and α parameters remained global). We fit each model in a univariate framework using the package OUwie (Beaulieu and O'Meara, 2014) and assessed model fit by comparing the mean Akaike information criterion (AIC_c). Finally, we conducted a separate univariate assessment of the quantitative relationship between maximum plant height and defense. All phylogenetic comparative analyses were run using R version 3.3.2 (R Development Core Team, 2015).

RESULTS

Our initial HPLC analysis of four *Asclepias* spp. revealed typical concentrations of cardenolides in *Asclepias* (≤7 mg/g cardenolide on a leaf dry mass basis), and trace concentrations in the five *Pachypodium* spp. (Fig. 3; difference between the genera

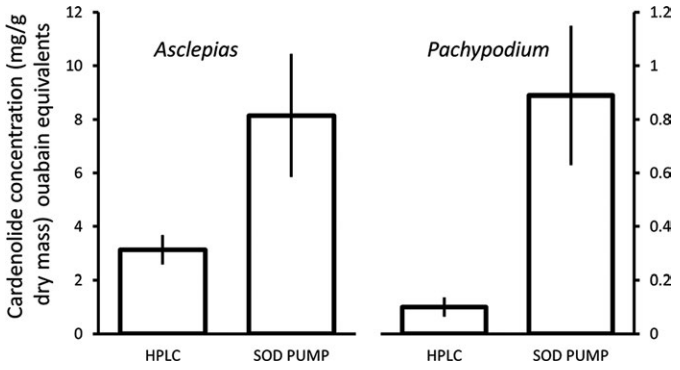

FIGURE 3. Two ways of estimating cardiac glycosides across four *Asclepias* species and five *Pachypodium* species. Although *Pachypodium* spp. have, on average, an order of magnitude lower cardenolide concentration than *Asclepias* spp. (when measured by HPLC), their extracts are proportionally more inhibitive (when activity is measured on the sensitive porcine Na^+/K^+-ATPase).

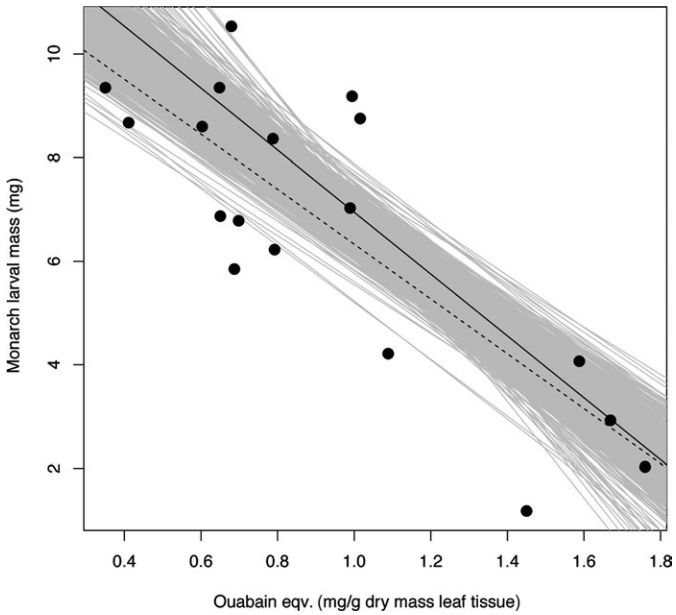

FIGURE 4. Impact of *Pachypodium* cardiac glycosides (measured as inhibition of the porcine Na^+/K^+-ATPase, in ouabain equivalents) on monarch caterpillar growth. Each point is a *Pachypodium* taxon (see Fig. 2 and Appendix 1). Shown are the raw (dashed) and phylogenetically independent (solid) slopes, the latter calculated over 1000 phylogenies from the posterior distribution.

$F_{1,85}$ = 67.92, P < 0.001). Based on our standard criteria of absorbance at 220 nm, we identified two putative cardenolides in *Pachypodium* extracts, providing the first evidence of these compounds in *Pachypodium* (Appendix S1). Analysis of inhibition of the highly sensitive porcine Na^+/K^+-ATPase revealed significantly higher estimates of cardiac glycosides across species in both genera ($F_{1,85}$ = 16.98, P < 0.001), but, more importantly, we found a statistical interaction between genus and assay method ($F_{1,85}$ = 67.92, P < 0.001). This result demonstrates that *Pachypodium* cardiac glycosides were proportionally more toxic (8.6× higher than predicted by HPLC) than *Asclepias* cardenolides (2.6× higher than predicted by HPLC) (Fig. 3).

The 18 *Pachypodium* taxa assayed showed fivefold variation in their capacity to inhibit the porcine Na^+/K^+-ATPase (Appendix 1). This inhibition showed low but statistically significant phylogenetic signal (Blomberg's K = 0.372, P = 0.03; Fig. 2). The best-fitting model for the evolution of sodium pump inhibition was a BM model of evolution, with no improvement from considering geographic origin, growth form, or habitat association (Appendix S4). Maximum height of *Pachypodium* spp. predicted Na^+/K^+-ATPase inhibition (r^2 = 0.38, $F_{1,16}$ = 9.81, P = 0.006), but this pattern weakened after phylogenetic correction (r^2 = 0.17, $F_{1,16}$ = 3.37, P = 0.085) (Appendix S5).

Estimates of Na^+/K^+-ATPase inhibition strongly predicted monarch caterpillar growth in our bioassay (Fig. 4; raw linear regression R^2 = 0.661, P < 0.001; phylogenetic gls across all phylogenies, mean ± SD, R^2 = 0.46 ± 0.05, P = 0.002 ± 0.002). Our analysis of the inhibitory effects of *Pachypodium* and *Asclepias* extracts on the monarch and porcine ATPases revealed that all main effects in the model were highly significant (P < 0.001). Most importantly, the monarch ATPase showed 100-fold greater resistance to milkweed extracts compared to the porcine ATPase; nonetheless, the *Pachypodium* extracts had nearly equal inhibitory effects on the two ATPases (results for each species are reported in Fig. 5; mean [± SE] log concentration for IC50 for the two plant genera: *Pachypodium* on porcine ATPase, −1.91 ± 0.08; *Pachypodium* on monarch ATPase, −1.64 ± 0.08; *Asclepias* on porcine ATPase, −2.80 ± 0.10; *Asclepias* on monarch ATPase, −1.78 ± 0.10; plant genus × ATPase source, $F_{1,68}$ = 17.29, P < 0.001). Note that higher values (less negative) represent higher cardiac glycoside concentrations needed to inhibit the Na^+/K^+-ATPase to the same level (50% inhibition).

DISCUSSION

Water-storing succulent plants are typically defended both by physical barriers, such as spines, and by chemical toxins such as alkaloids and cardiac glycosides. The culturally long-known toxicity of *Pachypodium* led us to investigations of defense mechanisms (Rowley, 1999). Cardenolides are widely produced in the Apocynaceae, whereas they occur only sporadically in other botanical families. A preliminary, macro-scale phylogenetic analysis across the Apocynaceae revealed cardenolides in 27% of the surveyed genera, with several independent origins of cardenolide production (Agrawal et al., 2012). In addition, some 75% of the surveyed genera contained alkaloids, with differing types, including steroidal, indole, and pyridine alkaloids. Burzynski et al. (2015) surveyed pyrrolizidine alkaloids (PAs) across the Apocynaceae and reported that distinct classes of PAs were produced in three lineages of the family. The Apocynaceae appear to be particularly diverse in their chemical defenses, these compounds typically being coupled with latex exudation and less commonly with spines (Rowley, 1999; Endress and Bruyns, 2000; Agrawal et al., 2008; Agrawal and Konno, 2009). The insects associated with the Apocynaceae are typically highly specialized and have a diversity of mechanisms to cope with such barriers to feeding (Dussourd and Eisner, 1987; Trigo et al., 1996; Dussourd, 2009; Dobler et al., 2012; Petschenka et al., 2013b; Burzynski et al., 2015; Agrawal, 2017).

As noted, the toxicity of *Pachypodium* has long been known, beginning with the widespread use of extracts on poison arrows

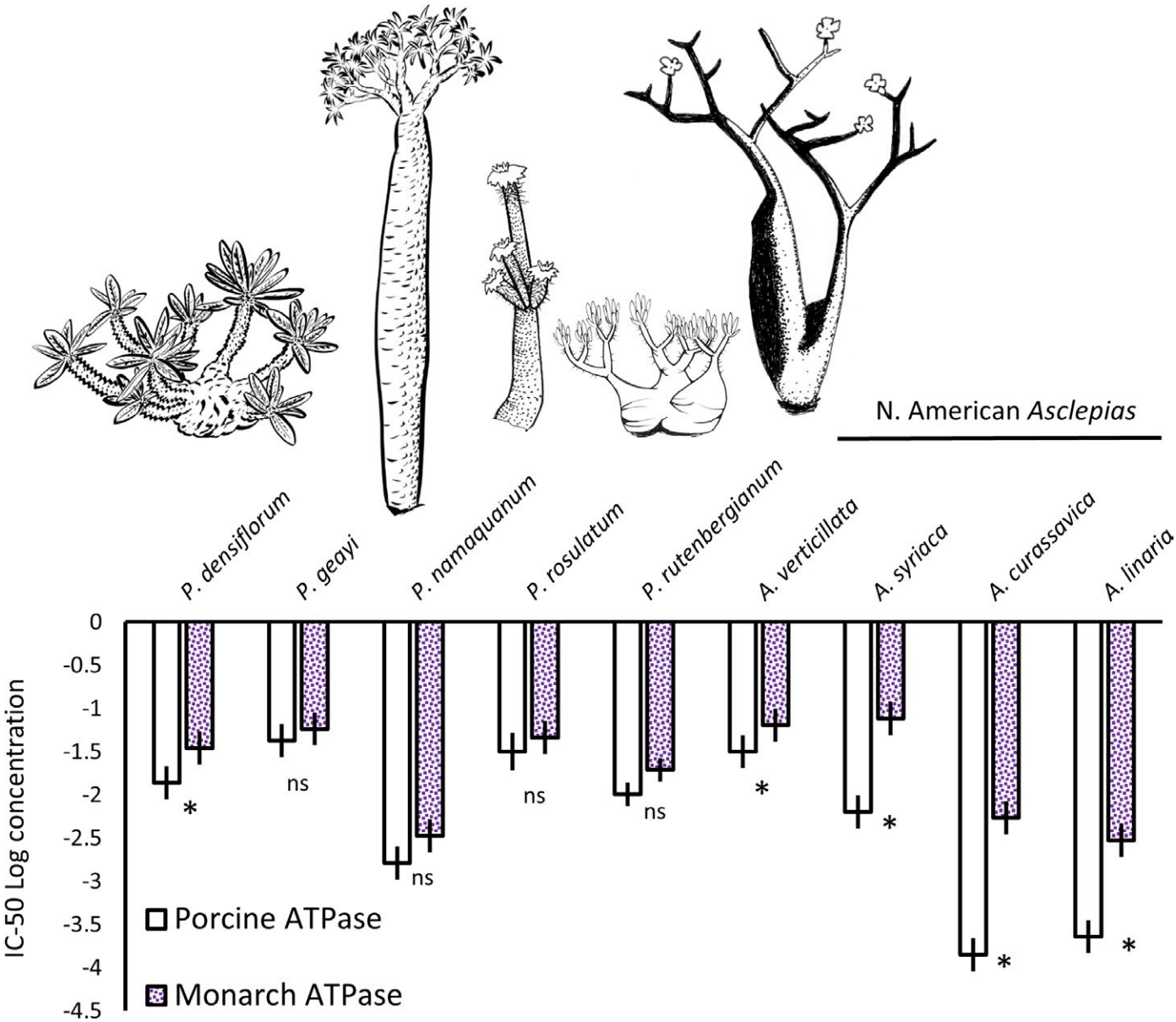

FIGURE 5. The difference in inhibitory impacts of *Pachypodium* and *Asclepias* plant extracts on the cardenolide-adapted monarch Na^+/K^+-ATPase vs. highly sensitive porcine Na^+/K^+-ATPase (presented as log concentration necessary to cause 50% inhibition). Overall, *Pachypodium* is equally inhibitive of the two enzymes, whereas the monarch ATPase is 100-fold more resistant to *Asclepias* extracts than the porcine ATPase (see text for full statistical model). Asterisk indicates $P < 0.05$ in one-way ANOVAs for each species. Note that because raw cardiac glycoside concentrations were under a value of 1, the presented log transformed values are negative. Higher values (less negative) represent higher cardiac glycoside concentrations needed to inhibit the Na^+/K^+-ATPase to the same level (50% inhibition). Illustrations by Bonnie McGill and Marjorie Weber.

by native people in southern Africa (Hoffman and Bigger, 1980; Neuwinger, 1998). During the German colonial occupation of southwestern Africa, Helly (1905) studied poison arrows that had been coated with extracts of *P. lealii* roots from what is now northern Namibia. These extracts proved to have a pronounced impact on vertebrate cardiac function, as revealed by studies of frogs, rabbits, and dogs, and were dubbed "*Pachypodiin glycosides*." Some 50 yr later, Reichstein's steroid chemistry lab evaluated several *Pachypodium* spp. (Abisch and Reichstein, 1960; Meyer et al., 1964). Although lactones as well as traces of alkaloids were reported from *Pachypodium* spp., the authors concluded that cardenolides may be absent. They found an abundance of 2-desoxysugars, which are typical for cardenolides, but concluded that they may be diterpene glycosides.

Our results initially suggested relatively low concentrations of cardenolides in *Pachypodium* spp. when measured by HPLC, but these same plant extracts were more potent than expected when applied to the porcine Na^+/K^+-ATPase. *Pachypodium rutenbergianum*, in particular, showed a high level of toxicity in initial analyses. Across the genus, toxicity ranged over fivefold (Fig. 2 and Appendix 1), with some phylogenetic signal evident. For example, sister species from southern Africa *P. lealii* and *P. saundersii*

(which have divergent growth forms) were among the most toxic, and the three Malagasy subspecies of *P. rosulatum* were all on the low end of toxicity. Nonetheless, other pairs of close relatives, such as *P. namaquanum* and *P. bispinosum*, have substantially diverged. This pattern of phylogenetic signal coupled with divergence is the widely held expectation for defense evolution as adaptation results in distinct habitats and growth forms (Agrawal, 2007; Futuyma and Agrawal, 2009). We found evidence that taller *Pachypodium* spp. showed proportionally greater toxicity (inhibition of the Na^+/K^+-ATPase), although this relationship was not significant after phylogenetic correction. This finding is consistent with the hypothesis that more apparent species will invest more in defense (Malcolm, 1991; Agrawal, 2007).

Remarkably little is known about herbivory on *Pachypodium*. Midgley et al. (1997) described possible porcupine and baboon damage in the Richtersveld part of the Karoo in South Africa, although damage was most evident on *Aloe* spp., which share habitat with *P. namaquanum*. As for insect herbivory, only larvae of the oleander hawk-moth, *Daphnis nerii*, has been reported on *Pachypodium* in Madagascar (Attie et al., 2010). *Daphnis nerii* is a specialist on Apocynaceae, with various detoxification mechanisms (Abe et al., 1996; Petschenka and Dobler, 2009). Interestingly, *D. nerii* maintains a highly sensitive Na^+/K^+-ATPase but copes with polar and nonpolar cardenolides with passive and active mechanisms at its perineurial blood-brain barrier (Petschenka et al., 2013a). Thus far, it is unclear whether *Pachypodium* hosts a taxonomically rich fauna of insect herbivores, as do other New World and Old World Apocynaceae (Agrawal, 2017).

Asclepias, Pachypodium, and novel chemical ecology

Among milkweed herbivores in North America, a community has assembled that is convergent both behaviorally and physiologically (Dussourd and Eisner, 1987; Dobler et al., 2012). This group is taxonomically diverse (at least six insect orders) and feeds on all plant parts, from roots and shoots to phloem and seeds. Similar faunas have independently colonized Apocynaceae in North Africa and India (reviewed in Agrawal, 2017). Because genetic substitutions that modify cardenolide binding to the Na^+/K^+-ATPase have been found in many of these species (Al-Robai, 1993; Dobler et al., 2012; Petschenka et al., 2013b; Bramer et al., 2015; Dobler et al., 2015; Schneider et al., 2017), we decided to use the North American monarch butterfly as a model to understand the defense chemistry and mode of action of *Pachypodium* toxins. Indeed, the monarch is up to 200-fold more resistant to cardenolides than basal danaine butterflies, due to a few genetic substitutions in the sodium pump (Vaughan and Jungreis, 1977; Petschenka and Agrawal, 2015). We expected that if *Pachypodium* spp. contained typical cardenolides, monarchs would have substantial resistance to the compounds. Nonetheless, in our bioassay, we found tremendous variation in monarch growth depending on the species of *Pachypodium* extract that was painted on milkweed leaves, and this effect was strongly correlated with the inhibitory activity estimated from the highly sensitive porcine Na^+/K^+-ATPase.

If *Pachypodium* contains highly distinct cardiac glycosides, they may show dissimilar patterns of inhibition, and monarchs may show relatively little resistance to these compounds. In a related study, we have shown tremendous variation in the inhibition of sensitive and resistant (monarch) Na^+/K^+-ATPases by cardenolides with particular structural attributes (G. Petschenka, C.S. Fei, J.J. Araya, S. Schröder, B.N. Timmermann, A.A. Agrawal, unpublished data). Indeed, our analysis revealed strong resistance of the monarch Na^+/K^+-ATPase to cardenolide extracts from four *Asclepias* spp. (native to North America, typical food plants of the monarch), while *Pachypodium* spp. showed nearly equivalent inhibition of the sensitive and resistant Na^+/K^+-ATPases.

It has long been thought that cardiac glycosides are generalized toxins, given that their target site is the Na^+/K^+-ATPase, a highly conserved cellular enzyme in animals; nonetheless, cardiac glycosides are highly specific in that their mode of action seems restricted to inhibiting the Na^+/K^+-ATPase, and few other compounds are toxic by these means (Agrawal et al., 2012). It is now clear that both structural variation in cardiac glycosides and the extent of adaptation in insect herbivores will dictate the outcome of the toxin–target site complex (Petschenka and Agrawal, 2016; G. Petschenka, C.S. Fei, J.J. Araya, S. Schröder, B.N. Timmermann, A.A. Agrawal, unpublished data). In addition, novel compounds are emerging that inhibit the Na^+/K^+-ATPase, including sanguinarine, an isoquinoline alkaloid from poppies (Mackraj et al., 2008), and palytoxin, a fatty alchohol from marine organisms (Rossini and Bigiani, 2011). Perhaps most relevant to the present study, trisaccharides (containing lactones) have been found in roots of *Decalepis hamiltonii* (Apocynaceae), which have strong insecticidal activity derived from inhibiting the Na^+/K^+-ATPase (Rajashekar and Shivanandappa, 2017).

We speculate that *Pachypodium* has evolutionarily forged a distinct set of chemical compounds that inhibit the animal sodium-potassium pump and have potential to poison herbivores. As has been demonstrated in other systems, plants with novel chemistry can prove toxic, even to herbivores that are specialized on related plants (Berenbaum, 1983; Renwick, 2002; Davis et al., 2015). In several other plant–herbivore systems, coevolutionary interactions appear to have driven the escalation of plant defense toward novel chemical compounds that target specialized herbivores (Rodman et al., 1982; Berenbaum, 1983; Agrawal et al., 2009a, b; Becerra et al., 2009; Cacho et al., 2015; Edger et al., 2015). At this stage, it is unclear whether *Pachypodium* toxicity is a response to particular herbivores or is due to isolation in distinct habitats. The biotic and abiotic agents of selection on *Pachypodium* and the identity of its novel toxic compounds await further study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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APPENDIX 1. Species and sources of plant material used in this study. Most are from private collections as indicated. Nomenclature follows Burge et al. (2013). Species identity was checked by DNA sequencing (see text). Mean and standard error (SE) are presented for leaf cardiac glycosides, based on four or five replicates of each *Pachypodium* sp. in ouabain equivalents (mg/g dry mass) and inhibition of the porcine Na^+/K^+-ATPase. Maximum height represents values from natural populations. Species with maximum heights >3 m were considered "trees," while others were considered "shrubs."

Species	Source of plant material	Mean	SE	Maximum height (m)
P. ambongense	Kevin Spiers, Austin, TX	0.788	0.225	2
P. baronii	Maureen Massara, Naples, FL	1.015	0.225	3.5
P. bispinosum	Corona Cactus Nursery, Corona, CA	0.679	0.259	0.5
P. decaryi	Maureen Massara, Naples, FL	1.089	0.225	4
P. densiflorum	Kevin Spiers, Austin, TX; Bill Ballard, Gallipolis, OH	0.603	0.225	0.7
P. eburneum	Kevin Spiers, Austin, TX; Bill Ballard, Gallipolis, OH	0.698	0.201	0.4
P. geayi	Le Jardin Naturel, Saint Leu, Reunion; Kevin Spiers, Austin, TX	0.687	0.150	10
P. horombense	Bill Ballard, Gallipolis, OH	0.651	0.201	0.6
P. inopinatum	Maureen Massara, Naples, FL; Corona Cactus Nursery, Corona, CA	0.792	0.259	0.4
P. lamerei	Kevin Spiers, Austin, TX; Le Jardin Naturel, Saint Leu, Reunion	0.989	0.183	5
P. lealii	Maureen Massara, Naples, FL; Corona Cactus Nursery, Corona, CA	1.669	0.318	10
P. namaquanum	Corona Cactus Nursery, Corona, CA	1.588	0.259	4
P. rosulatum ssp. *bicolor*	Maureen Massara, Naples, FL; Corona Cactus Nursery, Corona, CA	0.648	0.259	0.5
P. rosulatum ssp. *cactipes*	Bill Ballard, Gallipolis, OH; Le Jardin Naturel, Saint Leu, Reunion	0.351	0.159	0.8
P. rosulatum ssp. *rosulatum*	Maureen Massara, Naples, FL	0.411	0.318	2.5
P. rutenbergianum	Le Jardin Naturel, Saint Leu, Reunion; Kevin Spiers, Austin, TX	1.760	0.183	12
P. saundersii	Le Jardin Naturel, Saint Leu, Reunion	1.450	0.259	1.5
P. windsorii	Bill Ballard, Gallipolis, OH	0.994	0.225	1.5