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Phylogeny of kangaroo apples (*Solanum* subg. *Archaesolanum*, Solanaceae)

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Abstract Kangaroo apples, subgenus *Archaesolanum*, are a unique and still poorly known group within the genus *Solanum*. Here we aimed to reveal phylogeny, historical biogeography and age of diversification of *Archaesolanum*. We sampled all recognized species of the group and sequenced three chloroplast regions, the *trnT-trnL* spacer, *trnL* intron and *trnL-trnF* spacer to calibrate a molecular clock to estimate the age of the group. Distributional data were combined with the results of phylogenetic analysis to track the historical processes responsible for the current range of the group. Our analysis supported the monophyly of the kangaroo apples and the biogeographical disjunction between the two subclades within the group. Based on the divergence time estimates the most recent common ancestor of kangaroo apples is from the late Miocene age (~9 MYA). Based on the age estimate the common ancestors of the kangaroo apples are presumed to have arrived in Australia by long-distance dispersal. The two distinct lineages within the group have separated during the

aridification of the continent and further speciated in the brief resurgence of rainforests during the Pliocene.

Keywords Kangaroo apples · *Archaesolanum* · *Solanum* · Australia · Phylogeny

Introduction

Solanum L. subgenus *Archaesolanum* Bitter ex Marzell,¹ often called kangaroo apples, is composed of eight species occurring only in the SW Pacific region (Australia, Tasmania, New Zealand, Papua New Guinea). The group is characterized by its unique chromosome number ($x = 23$), possibly resulting from an aneuploid loss from a polyploid ($x = 24$) ancestor [1]. Interestingly, species have generated a further secondary polyploid series. Consequently, these diploids ($x = 46$, e.g. *S. aviculare*) could be better regarded as “tetraploids”—in terms of the $x = 12$, typical basic chromosome number of the genus—while tetraploids of the group ($x = 92$, e.g. *S. multivenosum*) are therefore better understood as “octoploids”. In other words, it is presumed that the early ancestor of this group has gone through a simple ploidy increase accompanied by a chromosome loss and then the duplication has been repeated. This unique feature makes *Archaesolanum* particularly interesting from an evolutionary standpoint. However, genetic relationships and how this interesting chromosome number has developed are unknown. In addition, phylogeny of this group has not yet been studied utilizing molecular tools despite the fact that many molecular studies on phylogenetic relationships within the genus *Solanum* have included one or

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¹ Basionym of *Solanum* section *Archaesolanum* (Bitter ex Marzell) Danert.

two representative species from the subgenus [2–5]. Compared to other *Solanum* clades, evolutionary dynamics, biogeography, dispersal, radiation and genetic diversity of the group are very poorly known.

The earliest record of the kangaroo apples is by Forster [6] describing *Solanum aviculare* based on the specimen collected during Captain James Cook's second voyage to New Zealand. After Forster's first report, during the eighteenth and nineteenth century, at least three species were collected from coastal Australia or New Zealand and brought to Europe where they were cultivated in botanical gardens serving as material for confusing descriptions (for example [7, 8]) whilst others continuously published new names (e.g. [9, 10]) with varying success. These early studies have resulted in extensive synonymization and unsettled taxonomic concepts. As summarized by Spooner [11], species concepts in *Solanum* have been so controversial that even experienced taxonomists have provided different identifications for identical collections. However, many problematic cases have been settled by Baylis [12] and Symon [13].

Species concepts have been controversial within *Archaeosolanum* and also its status as a separate taxonomic unit has been ambiguous. Dunal [14] did not recognize the distinctiveness of the group and placed it under subsection *Dulcamara*. Later Bitter [15], in his last contribution to Gustav Hegi's book, published the name subgenus *Archaeosolanum*, typified by *S. aviculare*. However, Bitter died shortly before his work was published and it was edited by Marzell. The name given to the group suggests an ancient origin possibly attributed to the free oriented stamens and the presence of abundant stone cell mass in the fruit flesh. Since Bitter, the group has been recognized at sectional level [16]. It was elevated to subgeneric level by D'Arcy [17, 18], and again treated as a section [19] then once more as a subgenus [20]. In studies utilizing sequence level characters [3–5] the group has been distinguished as the *Archaeosolanum* clade. All treatments agree that its distinction from other members of the genus is obvious, and that the species form a unique and coherent group within *Solanum*. So far very little work has been done to characterize further subdivisions within the group. Gerasimenko [21] described three series, and this division, as modified, is also applied today [13].

The *trnT-trnF* region is located in the large single-copy regions of the chloroplast genome, approximately 8 kb downstream of *rbcL* [22]. This region consists of three highly conserved transfer RNA genes namely tRNA genes for threonine (UGU), leucine (UAA) and phenylalanine (GAA) [23]. These exons are separated by two intergenic spacers (*trnT-L* and *trnL-F*) while the *trnL* gene is split by a group I intron [24]. As the advent of molecular methods has revolutionized the field of plant systematics [25–31]

this region became widely used due to the high variability. We have chosen this region for our investigations because the evolution of the *trnT-L* has been thoroughly analyzed and is well understood [23] to calibrate a molecular clock. More recently, it was also shown that this region comprises more phylogenetic structure per informative character than *matK* [32] another widely used chloroplast region in phylogenetics.

In the present work we aimed to clarify the taxonomy of kangaroo apples and to investigate speciation processes by using chloroplast DNA sequences. We also estimated the age of the most recent common ancestor of the group using a molecular clock and used these dates to evaluate the biogeographical history of the group in Australia.

Materials and methods

Taxon sampling

Three accessions per species were analyzed for *Solanum aviculare*, *S. laciniatum* (Fig. 1a), *S. linearifolium* (Fig. 1b), *S. simile* (Fig. 1c, d), *S. symonii* (Fig. 1e), *S. vescum* (Fig. 1f) and two accessions for *S. capsiciforme*. Only one accession was sampled of the rare *S. multivenosum* with only few herbarium records. This is due to its restricted occurrence in the high altitude (>2,500 m) mountain ranges of Papua New Guinea where kangaroo apples have still been poorly collected. The outgroup exemplars from other *Solanum* subgenera and outside the genus were selected following the results of Weese and Bohs [4] and Olmstead et al. [33]. For the molecular clock analysis a further outgroup (*Ipomoea purpurea*) was included to represent the split between Solanaceae and Convolvulaceae following Paape et al. [34]. Further information about the terminals is summarized in Electronic supplementary Material 1.

Laboratory techniques

Total genomic DNA was extracted from 50 mg of fresh leaves following the protocol of Poczar and Hyvönen [35]. From the herbarium specimens extractions were made with the NucleoSpin 96 Plant Kit (Machery-Nagel). Absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) was measured for each DNA sample using the NanoDrop 2000 (Thermo Fisher Scientific, USA) spectrophotometer. Sample concentration was calculated by the NanoDrop nucleic acid application module using Beer's law, and assuming 50 ng cm/ml absorbance for dsDNA, A_{260}/A_{280} ratios averaged 1.79 ± 0.12 SD. Each sample was diluted to 20 ng/μl final concentration. The complete *trnT-trnF* chloroplast region was amplified in three overlapping fragments with the

Fig. 1 **a** Flowers of *Solanum laciniatum*; **b** *Solanum linearifolium*; **c, d** *Solanum simile*; **e** *Solanum symonii*; **f** *Solanum vescum*

universal primers designed by Taberlet et al. [36]. The $trnT^{UGU}$ - $5'trnL^{UAA}$ spacer region was amplified with primers A (5'-CAT TAC AAA TGC GAT GCT CT-3') and B (5'-TCT ACC GAT TTC GCC ATA TC-3'); $5'trnL^{UAA}$ - $3'trnL^{UAA}$ intron with primers C (5'-CGA AAT CGG TAG ACG CTA CG-3') and D (5'-GGG GAT AGA GGG ACT TGA AC-3'), and finally the $3'trnL^{UAA}$ - $trnF^{GAA}$ spacer region with primers E (5'-GGT TCA AGT CCC TCT ATC CC-3') and F (5'-ATT TGA ACT GGT GAC ACG AG-3'), respectively. Amplification reactions were performed in 50 μ l volumes containing: 25 μ l NFW (Nuclease Free Water), approx. 20 ng template DNA, 0.5 μ M of each primer, 0.2 mM dNTP (Fermentas, Lithuania), 5 μ l 10 $\times$ PCR buffer (1 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM MgCl₂, 50 mM KCl and 0.1% Triton X-100) and 0.5 U of DyNzyme II (Finnzymes, Finland) polymerase. All reactions were done in a MasterCycler ep96 (Eppendorf, Germany) with the following settings: 2 min at 94°C

for initial denaturation, 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 50°C, and 2 min extension at 72°C, followed by a final extension for 5 min at 72°C. Amplification products were separated on 1.5% agarose gels (GE Healthcare, UK) in 0.5 $\times$ TBE buffer (220 V, 0.5 h) and stained with ethidium-bromide.

Fragments excised from agarose gels or direct PCR products were cleaned with NucleoSpin Extract II Kit (Machery-Nagel, Germany) and cloned to JM107 competent *Escherichia coli* strains using ColneJET PCR Cloning Kit (Fermentas, Lithuania) and the Transform Aid Bacterial Transformation Kit (Fermentas, Lithuania). The procedure was carried out according to the manufacturer's instructions. Plasmids were extracted from the selected colonies holding the desired insert with NucleoSpin Plasmid DNA Kit (Machery-Nagel, Germany). All DNA sequencing was performed on an ABI 3730XL automated sequencer using ABI PRISM BigDye Terminator Cycle

Fig. 2 Distribution of kangaroo apples (subg. *Archaeosolanum*) in the South Pacific region.

a *Solanum aviculare*, **b** *Solanum capsiciforme*, **c** *Solanum laciniatum*, **d** *Solanum linearifolium*, **e** *Solanum multivenosum*, **f** *Solanum simile*, **g** *Solanum symonii*, **h** *Solanum vescum*

Sequencing Ready Reaction Kit v. 3.0 (Perkin-Elmer/Applied Biosystems, California, USA) with pJET1.2 forward and reverse primers. The sequencing of three separate plasmids per each analyzed terminal was done in both directions to detect possible ambiguities.

Sequence alignment and phylogenetic analysis

Sequences were aligned with ClustalW implemented in BIOEDIT [37] using the default parameters of a match score of 5, a mismatch penalty of 4, a gap open penalty of 15 and a gap extension penalty of 6.66. The matrix is available from TreeBASE (Submission ID 10328).

Phylogenetic analyses with parsimony as the optimality criterion were performed using Nona [38] within the Winclada [39] shell. Because sequences of species with multiple entries did not show any variation within species, only one for each species was included in the analyses, thus resulting in 31 terminals. Four different analyses were made with the following settings: hold* (number of trees held in memory, * denoting as many as the memory allows), mult 5000 (number of search replicates), hold/2 (number of randomly chosen starting trees per replicate), and using mult*max* (multiple tree-bisection-reconnection algorithm). The second analysis was performed with the same settings, only with an increased number of starting trees (hold/20). We employed also the parsimony ratchet [40] in two additional analyses with the following settings: 1000 replicates, two trees hold per iteration, 400 characters (ca. 20%) reweighted, and with amb-poly = (default setting of Nona: if any of the reconstructed states are shared between ancestor and descendant node, the branch is collapsed). Another ratchet analysis was performed with the same settings but with an increased number of trees held per iteration (hold/10), and with 600 characters (ca. 30%) reweighted.

Jackknife [41] support values were calculated with 10,000 replicates using the Mac OSX version of the program TNT [42].

Molecular clock and divergence time estimation

For the selection of the appropriate nucleotide substitution model we used jModelTest [43] to calculate the probabilities of changes between nucleotides along branches of phylogenetic trees using the bayesian information criterion (BIC). For the parsimony analyses the original combined chloroplast sequence matrix was pruned for molecular clock dating, keeping only one representative sequence from each species of subg. *Archaeosolanum*, since there was no intraspecific variation. This was done to shorten the computational time needed for each run. For this data

matrix the GTR + Γ (General Time Reversible + Gamma) was determined as the best-fitting statistical model. Tree topology, node ages and substitution rates were simultaneously estimated with Bayesian Markov chain Monte Carlo (MCMC) simulations using BEAST v. 1.5.2 [44]. We employed an uncorrelated and lognormal distributed relaxed clock (UCLD), implemented in BEAST, which allowed different branches of the trees to have independent clock rates, making no assumption about the correlation between substitution rates in the tree. The value of mean substitutions per million years (MY) was fixed at 0.0007, according to the estimates of previous studies concerning coding and non-coding regions of cpDNA [45, 46]. We assumed a constant rate of speciation per lineage and selected the Yule Speciation Process as the tree prior, which has been recommended for species-level phylogenetics [47]. Operators were auto-tuned and the starting tree was chosen to be randomly generated. Fossil dates were used as calibration points to reduce the bias and generate more accurate age estimations [48]. However, fossil records of the Solanaceae are very limited; the earliest ones for *Solanum*-like and *Physalis*-like seeds are from the mid-Miocene and for Convolvulaceae from the lower Eocene [49]. These records have been previously regarded as sufficiently reliable [34] but conclusive analyses about their relationships have not been made. The clades of interest were defined via most recent common ancestors (MRCA), and they could thus have varying taxon composition in the posterior. Normal distribution priors were applied to the calibration points at nodes: the MRCA of *Solanum* and *Physalis*, with a mean age of 10 MY and a standard deviation (SD) of 4 MY; and the MRCA of *Ipomoea purpurea*, with a mean age of 52 MY and an SD value of 5.2 MY to represent the split between Solanaceae and Convolvulaceae as a minimum age constraint [50]. The SD values represent the upper and lower bounds of geological epochs from which the fossils were obtained as previously proposed by Paape et al. [34]. Two separate runs were performed with 10 M generations sampling every 1 000th tree, with a burn-in of 1 M generations each. The results of the individual runs were checked for convergence and analyzed with Tracer v. 1.5 [51], then combined into one consensus log file with LogCombiner v. 1.5.2 [44], as recommended for phylogenetic MCMC analysis, instead of running single but considerably longer chains [52]. The effective sample sizes (ESS) for all estimated parameters were well above 100. The resulting trees were finally combined into one consensus tree with TreeAnnotator 1.5.2 [44] and edited with FigTree v. 1.3.1 [53]. In order to evaluate temporal variation in rates of diversification a lineages-through time plot was calculated based on BEAST estimates.

Geospatial analysis

GenGIS version 1.08 [54] was used to combine distributional data with the phylogenetic information obtained from the parsimony analysis. The location set (simple sites) file is available as Electronic Supplementary Material 2. This was done to track the historical processes that might be responsible for the current distribution of lineages as well as to analyze and visualize past events that might be inferred. To this end GenGIS provided a 3D graphical interface to combine georeferenced genetic data into a cartographic display to yield a clear view of the relationships between phylogenetic relationships and distribution. In the first step digital map data were obtained with GenGIS MapMaker [55] using the world maps provided by Natural Earth (<http://www.natureearthdata.com/>). The region of interest within the world map was selected and cropped in order to create georeferenced maps specific to the kangaroo apple data set. The location file was generated manually in a comma-separated format containing the taxon set with a unique location identifier and their decimal degrees of latitude and longitude. We used the consensus tree of the final parsimony analysis as an input phylogenetic tree. It should be noted that for *Archaeosolanum* all of the EPTs were completely congruent and thus the consensus tree was fully resolved. The digital elevation map (DEM) of the South Pacific region together with the location and phylogenetic tree file was loaded to GenGIS. To determine the optimal tree layout, GenGIS uses a branch-and-bound algorithm to find the optimal ordering of leaves and internal nodes of a tree that minimizes the number of generated crossings [54]. Crossings are the mismatches between tree ordering and sample sites measuring the fit between geography and phylogeny. Linear geographic axes were defined arbitrarily and used for the 2D transformation of cladograms. To examine the possible relationships between terminals and latitude we performed a linear regression analysis based on these transformations. This was done by employing a permutation test in 1000 iterations implemented in GenGIS to determine whether the fit of tree leaves to geography is significantly better than random, based on randomization of leaf nodes [54].

Historical biogeography

In order to explain the current distribution (Fig. 2) of kangaroo apples we inferred the probable location of an ancestral geographic range by performing event-based dispersal-vicariance analysis [DIVA; 56] and weight-ancestral area analysis [WAAA; 57]. Geographical areas were defined based on the distribution of endemic taxa and previously recognized regions in Australia for kangaroo apples by Symon [13]. The areas considered were the

Fig. 3 Consensus based on 138 equally parsimonious trees (EPTs) obtained in the four parsimony analyses (see the text for further details)

Coastal Southern West and South Australia (A), Inland Southern West and South Australia (B), South New South Wales (C), East New South Wales (D), East Victoria (E), Tasmania (F), South Victoria (G), Queensland (H), New Zealand (I), and Papua New Guinea (J). Distributional data were derived from the monograph of Symon [58] and from the Global Biodiversity Information Facility (GBIF) portal (<http://data.gbif.org/species/>).

For the WAAA analysis an area cladogram was obtained from the results of the BEAST and parsimony analyses (both resulted in identical topologies). The taxon-area cladogram was constructed by replacing the names of the terminals with the names of the areas in which they occur. Areas were treated as single transformation series. WAAA estimates ancestral areas using reversible parsimony, and weights areas in plesiomorphic branches more than apomorphic branches [57]. Therefore, gains and losses of an area are counted separately. The number of weight gain steps (GSW) and weight loss steps (LSW) on the area cladogram were calculated for each area at each node. The

final probability indices (PI) were calculated as the ratio of GSW and LSW for every area.

Input Nexus files for DIVA 1.2 [56] were evaluated manually and contained different area characters as absent (0)/present (1) for each species. DIVA assumes vicariance as the default option of speciation and counts steps for dispersal without signing any cost for vicariance. We constrained the distributional areas to include a maximum of four areas because increasing the number beyond this would provide results with innumerable combinations.

The distributions of climatic conditions affecting the group were analyzed and visualized using SEEVA v.0.33 [59]. Data files from SEEVA were prepared in an Excel file, containing the distribution presence (1)/absence (0) matrix of species in four predefined climatic zones [semi-arid (A), temperate (B), subtropical (C) and tropical (D)]. Areas were delimited based on the updated Köppen-Geiger climate map of Australia [60] and the distributional data of the species [13]. The Excel file together with the NEXUS file containing the pruned phylogenetic tree used previously for DIVA was loaded into SEEVA. Impact indices were calculated for each node on the tree according to Heiberg and Struwe [61]. The percentages of climatic zone categories in each sister group were mapped onto the phylogenetic tree.

Results

Phylogeny and age estimations

The parsimony analysis resulted in 138 distinct equally parsimonious trees of length 853 steps (Fig. 3). The values of consistency [62] and retention [63] indices were 0.85 and 0.78, respectively. The consensus [64] tree based on the 138 trees did not differ from the one based on 16 trees obtained in the first analysis. Both the parsimony and BEAST analyses resulted in congruent and completely resolved phylogenies of the kangaroo apples. The topology of the outgroup nodes was largely consistent with previously published phylogenetic trees [3–5]. The results are illustrated in the chronogram (Figs. 4, 5). The estimated value of the standard deviation of the uncorrelated log-normal relaxed clock (ucl.d.stdev) was 1.537 (95% HPD 1.104–1.959) while the coefficient of variation was 1.909 (95% HPD 0.967–2.864). These values indicate the presence of substitution rate heterogeneity across the tree and that the chosen relaxed clock model is the most appropriate. The Yule prior indicating the birth rate (i.e., speciation rate) was 0.071 (95%HPD 0.039–0.106). The tree separates two well resolved clades: i) a *Similia* clade (*S. simile*, *S. symonii*, *S. capsiciforme*); ii) an *Avicularia-Laciniata* clade with two subclades composed of *S. aviculare*,

S. laciniatum, *S. multivenosum*, and *S. linearifolium* plus *S. vescum*. All of these clades had high Bayesian posterior probabilities, with values ranging from 0.9008 to 1.0. The MCMC runs gave an estimated tMRCA of 17.95 MY (95% HPD 12.41–23.86) for the genus *Solanum* and 8.85 MY (95% HPD 4.45–14.03) tMRCA for the subgenus *Archaeosolanum*. The separation of Convolvulaceae and Solanaceae was estimated to be 61.95 MY (95% HPD 38.91–91.59).

Geospatial analysis

Georeferenced trees were drawn in two (Fig. 6) and in three dimensions to visualize the ecological and phylogenetic relationships among samples collected from different sites. The three dimensional tree and a video of this reconstructions is provided as Electronic Supplementary Materials 3 and 4. In order to find the optimal matches between the obtained phylogenetic tree and geography we defined geographical polylines in different orders and calculated *p*-values expressing the significance of each choice. When geographical locations were mapped on the parsimony tree, a global optimum of 20 crossings was observed for the *Archaeosolanum* clade. Defining a strict west-east (WE) axis, a permutation test on the cladogram yielded a *p*-value of 0.00095. Comparing this to the typical $\alpha = 0.05$ threshold level the null hypothesis can be rejected, indicating that geography is indeed an explaining factor for phylogeny of kangaroo apples. However, as yet, the program GenGIS does not allow broad testing of encoded hypotheses with automatic polyline enumeration. This is why we conducted permutation tests manually on a larger proportion of different linear axes. All of these defined in several directions, yielded *p*-values far greater than the 0.05 threshold level with observed crossings between 30 and 53, higher than the strict WE axis. The only significantly fitting orientation was a north-west/south-east (NW/SE) axis (24 crossings, *p* = 0.001). As the key principle in the tree construction through the defined linear geographical axis results in a dimensional gradient of similarity, the extent of data fit is expressed by the goodness of fit where mismatches are leading to crossings between the linking lines [54]. The tree with the smallest number of crossings represents the best fitting hypothesis between geography and phylogeny. Accordingly, the strict WE axis (20 crossings) applies better to our dataset than the NW/SE axis (24 crossings). A closer inspection of subclades within the selected WE polyline was conducted to understand the relative contribution of geography and habitat. The permutation test inferred from 1000 iterations was not significant in the case of the *Similia* subclade (6 crossings, *p* = 0.535) but yielded significant results with the *Avicularia/Laciniata* subclade (10 crossings, *p* = 0.047). Within

Fig. 4 Bayesian chronogram from the BEAST analysis. Geological time-scale abbreviations: PL Pliocene, P Pleistocene

Fig. 5 Bayesian chronogram of the subgenus *Archaeosolanum*. Geological time-scale abbreviations: PL Pliocene, P Pleistocene

Fig. 6 Geospatial representation of the phylogeny of the subgenus *Archaesolanum*. The optimal tree layout with 20 crossings is shown, with coloured branches and leaves, according to terminals taxonomic identity

this major clade permutation tests were also made for the two smaller subclades, one composed of *S. multivenosum*, *S. laciniatum* and *S. aviculare* (2 crossings, $p = 0.845$) and the other formed by *S. vescum* and *S. linearifolium* (3 crossings, $p = 0.668$), but neither of them had statistical significance. The SEEVA analysis was (Fig. 7) mostly congruent with the results GenGIS. However, impact indices were not so robust. In general the correlation between the analyzed environmental variables can be regarded as representing trends and tendencies within phylogenetic lineages, and not as the definite cause of lineage divergence.

Historical biogeography of kangaroo apples

The DIVA and WAAA results were largely congruent. The inferred ancestral areas are shown in Fig. 8 (results are summarized in Table 1). The DIVA reconstruction required at least 14 dispersal events to explain the present-day distribution of the group when the maximum number of areas was restricted to four at each node. There were four equally optimal reconstructions. Both DIVA and WAAA indicate that the most likely ancestral area was in Australia and New Guinea, and indicates a dispersal event to New Zealand. Three vicariance events are indicated in *Archaesolanum*, one separating the *Similia* and *Avicularia-*

Laciniata clades, another connected to *S. multivenosum* (resulting in further speciation), and one separating *S. vescum* + *S. linearifolium*.

Discussion

Monophyly and relationships within subgenus *Archaesolanum*

The results of the BEAST analysis were congruent with the parsimony analysis and supported a robust topology for a monophyletic *Archaesolanum* clade. At this point we propose to maintain *Archaesolanum* at the subgeneric level following Bitter's [15] original conspectus in concordance with D'Arcy [17, 18] and Hunziker [20], until higher level relationships of the whole genus are resolved and the closest relatives of the group are determined.

Analysis of the molecular data generated here separates two well-supported subclades within kangaroo apples. The *Similia* clade with *S. capsiciforme* + *S. symonii* + *S. simile*, and the *Avicularia-Laciniata* clade, further divided into separate *S. vescum* + *S. linearifolium* and *S. laciniatum* + *S. aviculare* + *S. multivenosum* subclades. Based on morphological characters Symon [13] also recognized a separate *Avicularia-Laciniata* clade, but *S. aviculare* was a sister species of *S. multivenosum*. In Symon [13] a separate *Similia* clade was not accepted because *S. capsiciforme* did not have a consistent position in the most parsimonious trees, either occupying a basal position relative to all other members, or else forming a clade with *S. simile* and *S. symonii*. Poczei et al. [5] separated *S. simile* from *S. aviculare* and *S. laciniatum* and found evidence to suggest the existence of two separate subclades within *Archaesolanum*. The topology obtained in the present analysis is congruent with those obtained in a pilot study based on multi-locus genetic markers [65]. However, the position of *S. multivenosum* was not determined because no material of this taxon was available. The position of *S. multivenosum* within *Archaesolanum* has been ambiguous since its description [66]. According to our results an early split between *S. aviculare* and *S. laciniatum* dates back to 3.5 MY, while a more ancient (6.1 MY) split separated *S. vescum* + *S. linearifolium*, the other members of the *Avicularia-Laciniata* clade. The relationships discussed above were proposed by Symon [13] who examined the surfaces of seeds with scanning electron microscopy (SEM). This analysis led him to postulate a group of *S. capsiciforme* + *S. symonii* + *S. simile* and a separate *S. aviculare* + *S. multivenosum* + *S. laciniatum* group with *S. vescum* + *S. linearifolium* in an intermediate position. The morphological characters together with the robust topology presented here

Fig. 7 Differences in distribution of climatic variables through the phylogeny of the subgenus *Archaeosolanum*. The numbers are impact indices for each node (see the text for further details); the bars indicate percentage of categories present in each group

Fig. 8 Mapping ancestral distribution of the subgenus *Archaeosolanum*. Blue letters (above lines) represent all alternative distributions at each node according to DIVA, while red letters (below lines) show WAAA results. Brackets indicate areas with lower probability indices. Letters right to each terminal indicate present day distribution

do not agree with the scheme of series proposed by Gerasimenko [21]. However, the recognition of a separate group of ser. *Similia* (*S. simile*, *S. symonii*, *S. capsiciforme*) seems to be valid while the separation of ser. *Avicularia* (*S. aviculare*, *S. cheesmanii*, *S. baylisii*, *S. brisbanense*²) from ser. *Laciniata* (*S. laciniatum*, *S. vescum*, *S. linearifolium*) seems unwarranted. Based on the phylogenetic hypothesis we present, ser. *Laciniata* would be paraphyletic. According to our results a new taxonomic scheme

² *S. brisbanense* Geras. is now recognized as synonym of *S. aviculare*; while *S. cheesmanii* Geras. refers to the white flowered (var. *albiflorum*) variety and *S. baylisii* Geras. to the broad leaf (var. *latifolium*) mutant of *S. aviculare*.

of taxa. Areas abbreviated are: Coastal Southern West and South Australia (A), Inland Southern West and South Australia (B), South New South Wales (C), East New South Wales (D), East Victoria (E), Tasmania (F), South Victoria (G), Queensland (H), New Zealand (I), and Papua New Guinea (J)

should be devised for further subdivision of *Archaeosolanum*.

Questions concerning the natural variation within this group and the status of previously published species and intraspecific taxa have not been intensively studied and remain open even after our analyses. The controversy is due to the studies based on the collections of Kondratenko and Kibaltchich in 1968. Many intraspecific names have been published based on material possibly originating from these collections [67–70]. However, they were never validly published as the descriptions lack Latin diagnoses and type specimen citations [71], thus not fulfilling the criteria of the International Code of Botanical Nomenclature [72].

Table 1 Mean posterior estimates of divergence times (MY) in the *Archaeosolanum* clade using BEAST, with the results of the ancestral area reconstructions using event based dispersal-vicariance analysis (DIVA) and weighted ancestral area analysis (WAAA)

Node	Age estimates Mean (MY)	DIVA	WAAA										Bayesian	
			A	B	C	D	E	F	G	H	I	J	PP	PP
0	8.85	ADFJ AEFJ ADIJ AEIJ	1.15	0.66	0.18	0.58	0.33	0.39	0.12	0.12	0.20	0.33	1.0	1.0
1	6.1	DFJ EFJ DIJ EIJ	0.17	–	–	1.51	1	1	0.17	0.17	0.33	0.66	0.99	0.99
2	3.5	FJ IJ	0.33	–	–	0.33	–	1	0.33	0.33	1	0.33	0.90	0.90
3	0.9	F, I	1	–	–	1	–	∞	1	1	∞	1	1.0	1.0
4	3.0	D, E	–	–	–	∞	∞	1	–	–	–	–	0.99	0.99
5	6.4	A	∞	3	0.33	0.33	–	–	–	–	–	–	0.90	0.90
6	3.2	A	∞	1	1	1	–	–	–	–	–	–	0.99	0.99

Node numbers refer to Fig. 7. Posterior mean ages are shown in millions of years before present. DIVA reconstructions are shown for optimality criteria maxareas = 4. WAAA reconstructions are shown with probability indices (PI). Biogeographical areas are indicated by capital letters according to Fig. 7. Area codes are discussed in the Materials and Methods section

In addition, these varieties would be better treated as horticultural forms and most of them (e.g. *S. baylisii*, *S. brisbanense*) have been synonymised by the global Planetary Biodiversity Inventory: *Solanum* Project [73]. On the other hand, these publications undoubtedly indicate that there must be considerable, and as yet unexplored, variation within some species of the group, which should be investigated further and documented properly.

Implications on higher level relationships:
an unresolved case

The unknown origin and lack of plausible common ancestors of *Archaeosolanum* and other species of *Solanum* have been previously recognized [3, 74]. However, some attempts have already been made to find even distant relatives of the group utilizing molecular markers. The first studies based on chloroplast restriction site variation and *ndhF* sequences, sampling only *S. aviculare* and *S. lacinatum*, indicated monophyly and a basal position for subg. *Archaeosolanum* [75], and relationships to other clades composed of members of sect. *Solanum*, *Dulcamara* and *Jasminosolanum* [76]. Bohs [3], using a broader taxonomic sampling for the *ndhF* gene, also supported the basal position of this group, with the closest relatives remaining unsettled. The latest analysis based on three genes (*ndhF*, *trnT-F*, and *waxy*) representing the broadest sampling of *Solanum* to date, indicated a surprising close relationship of *Archaeosolanum*, *Normania* and the so called African non-spiny clade [4]. However, this analysis included only two species of kangaroo apples. The present analysis including all recognized species of the group confirms the close relationship of the clades *Archaeosolanum* and *Normania* despite the great morphological differences between the two groups. However, the topology could still be misleading due to very limited sampling of both *Normania* and the African Non-Spiny clades. The *Normania* clade comprises only three species and thus the error, if due to small sampling, is most likely due to the inadequate sampling of the African non-spiny group.

Finding the higher-level relatives of kangaroo apples would be a problematic task. If the present conclusions and those by Weese and Bohs [4] are correct the closest relatives are to be found in *Normania*, or in the African non-spiny group. Obtaining good material for the Macaronesian *Normania* group is relatively difficult, because of the extreme rarity of these plants. For example only two living plants have been found from *Solanum nava* Webb & Berth. Since its original description [2, 77]. On the other hand, the African non-spiny clade is also poorly characterized both morphologically and at the molecular level. It is obvious that in order to find the closest relatives of kangaroo apples a more thorough sampling is needed of both of the groups

given above. Symon [13] suggested that the closest relatives of *Archaesolanum* are to be found within subg. *Solanum*. This is consistent with the fact that the African non-spiny clade is composed of members from sect. *Afrosolanum*, *Quadrangulare* and *Benderianum*, all belonging to subg. *Solanum*. In summary, our current sampling is not sufficient to settle the controversy concerning the closest relatives of *Archaesolanum*.

Reaching Australian shores: vicariance or long-distance dispersal?

Vicariance hypotheses have been challenged by previous botanical and molecular studies which aimed to study the historical biogeography of Solanaceae [18, 33, 76]. The most widely accepted hypothesis by Olmstead and Palmer [76] on the biogeography of *Solanum* species assumes that *Archaesolanum* presents an ambiguous case, either representing an early dispersal event in the genus, or a plausible case of vicariance dating to a time preceding the separation of South America and Australia. Our divergence time estimates suggest that the most recent common ancestor of kangaroo apples is from the late Miocene (~9 MYA). The observed split of the subgenus is unlikely to be the result of the break-up of Gondwanaland, since the division began already in the Jurassic, 180–150 MY ago [78]. The separation of Australia from Antarctica started in the late Cretaceous (90 MYA) and was completed in the late Eocene (35 MYA) with the opening of the South Tasman Sea [79]. However, some authors suggest a much earlier (~50 MYA) separation [80]. This geological break-up of the southern continent does not fit with our estimate dating back only to the late Miocene. Because, South America and Antarctica separated 30–28 MYA, while the distinct Australian continent collided with the Asian Plate (10 MYA; see [81]). These geological events together with our divergence estimates indicate that kangaroo apples probably do not represent a plausible case of vicariance dating to a time preceding the separation of South America and Australia as previously proposed [76]. These are in line with the hypothesis of D'Arcy [18] suggesting that the continental break-up was too early to have carried *Solanum* precursors. Vicariance requires that speciation and the corresponding fragmentation occur simultaneously, and thus information concerning absolute timing of speciation events is crucial for evaluating such scenarios [82]. However, molecular clock dating has been criticized as being inaccurate for a variety of reasons (see [83]). One of the major faults could be the inclusion of fossil records with ambiguous taxonomic affinities as clearly presented by Crepet et al. [48]. However, assuming that the presence of *Archaesolanum* in Australia is due to vicariance would challenge not only our molecular clock analyses but also

those presented for larger sampling of angiosperms [84] or those concentrated especially on Solanaceae [34]. Therefore, long-distance dispersal (LDD) with subsequent speciation is the most likely explanation for the occurrence of kangaroo apples in the SW Pacific. This might have taken place through trans-oceanic transmission by migrating birds, because only a few intercontinental plant disjunctions can be explained by water- or wind-mediated transports [85, 86], while other studies suggest that long-distance plant dispersal by birds is by far the most important vector [87]. However, not many birds are capable of retaining seeds for such a long time, although some studies report that viable seeds have been recovered from the gizzards of migratory birds after 200–360 h; for example, viable seeds of *Rhus glabra* L. have been recovered after they were in the digestive tract of a killdeer (*Charadrius vociferus* L.) for more than 14 days [88].

The soft and sticky, fallen ripe fruits of kangaroo apples are known to be eaten by birds [13, 89], which might support a long-distance dispersal scenario. Although, there is very little information available on exactly which bird species are responsible for their distribution. The first record of fruit predation by birds was made by Forster [6], which inspired him to give the telling name “*Aviculare*” (bird dispersed) for the first described species of the group. Later it was confirmed by other authors that birds play an important role in the distribution of seeds among the group [13, 89] especially in the case of species having red or orange colored fruits (e.g. *S. aviculare*, *S. laciniatum*) which are attractive to frugivorous birds. Previous aviary experiments with different plant species have demonstrated that migrant birds often exhibit color preferences, when factors such as taste, nutrition, and accessibility to food sources are equal [90]. Fruit colors as red, orange and yellow are commonly considered to increase the conspicuousness of a ripe fleshy crop and attract birds that eat fruits and disperse the enclosed seeds [91, 92]. However there are some records which indicate that mammals (e.g. rabbits, dingos) and macropods (e.g. wallabies, quokkas) may also be important for local dispersal [93].

Regarding the long-distance dispersal hypothesis, specific fruit traits might have played a role in the arrival of the group to Australia. The fruits of all kangaroo apples contain a relatively conspicuous and abundant proportion of stone cell aggregates. However, stone cell granules are commonly also present in some other groups of *Solanum* (e.g. sect. *Solanum* or sect. *Pachyphylla*; see [94]). The quantity and quality of these may vary from species to species as affected by environmental conditions. The anatomy and distribution of stone cells have been repeatedly studied (e.g. [16, 94]), but no final interpretation regarding their function has been presented. According to our divergence time estimate the character of abundant

stone cell formation seems to be an ancient trait in *Archaeosolanum*. The abundant stone cell mass produced by kangaroo apples might have had an important role in bird dispersal by protecting the seeds in the gizzard, or perhaps even by helping seeds to adhere to birds' legs or plumage.

Intercontinental dispersal from the African continent to Australia seems unlikely at first sight, considering that these areas are separated by more than 8,000 km of open ocean surface. Knowing that the shores of different continents have always been linked since the evolution of migratory birds [95]; makes this assumption more likely. These birds regularly travel across landmasses and they might carry internally or externally attached seeds from Africa/South America to Australia or even to New Zealand [86, 87]. A similar case of intercontinental dispersal of mucilaginous *Lepidium* L. seeds was demonstrated by Carlquist [95]. Mummenhoff et al. [96] using chloroplast *trnT-F* and nrDNA ITS sequences concluded that these Australian *Lepidium* s.s. (Brassicaceae) species were developed through allopolyploidization following not only one but two separate trans-oceanic dispersals from California and Africa to Australia and gave birth to the development of 26 modern species during the Quaternary. In another study based on also *trnL-F* chloroplast sequences and AFLP analysis the origin of a disjunct Australian *Microseris* D.Don (Asteraceae) lineage was revealed [97, 98]. It was concluded that Australian *M. lanceolata* (Walp.) Sch.Bip. is the outcome of a single introduction of a new hybrid formed in North America.

Other potential candidates as trans-oceanic long-distance dispersal events from Africa and South America to Australia are also known from the Solanaceae. The ancestors of *Lycium australe* F.Muell (Solanaceae) are assumed to have arrived from Africa [99, 100]. In this genus bird dispersal probably plays an important role, because *Lycium* L. species have relatively small succulent, orange, red berries which are very attractive to birds. Because of this feature at least three long-distance dispersal events are assumed to have occurred in the genus, resulting in the current range from North America to the Pacific Islands (e.g. Hawaii) as well as to South Africa and more interestingly from South Africa to Australia and possibly to the Eurasian region [99]. The single African taxa *Nicotiana africana* Merxm. is member of sect. *Suaveolentes* an Australian group of genus *Nicotiana* L. [101]. A recent study by Chase et al. [102] based on nrDNA ITS sequences and genomic in situ hybridizations (GISH) concludes that the monophyletic species of *Nicotiana* were derived from an allotetraploid progenitor from South America which reached the Australian continent by a single long-distance dispersal event. Unfortunately, this hybrid species left no trace in the area of origin. The mentioned examples outline that transoceanic long-distance dispersal events are often accompanied

with hybridization (e.g. *Lepidium*) and/or allopolyploid formation (e.g. *Nicotiana*). This might open the possibility of multiple origins for kangaroo apples. It seems reasonable to assume that kangaroo apples were developed in a plausible hybridization/allopolyploidization event. The question is when and where did this happen: were they formed by multiple introductions from separate continents? Did the characteristic chromosome formation happen in the new Australian environment or in the place of origin? These questions can be answered by revealing the origin of the most recent common ancestor of the group, but until that these questions will remain open.

Australian east–west disjunction

Our results indicate that kangaroo apples most likely diversified in the Miocene, thus representing an Australian floral element that arrived after the isolation of the continent and then radiated. During the Late Eocene, as the continent moved north, the Australian climate dried out, with more pronounced seasonality. This resulted from the action of the circumpolar currents and the formation of ice caps in the Antarctic [103]. The climate became cooler and drier, and kangaroo apples radiated to more humid habitable areas (SE and SW Australia; NW Queensland). The lineage through time plot (Fig. 9) suggests that the diversification of kangaroo apples proceeded steadily in the early stages of their evolution. Two new lineages then emerged in the Early Pliocene, and they diversified again in the Early/Late Pliocene. This coincides with the continental aridification of Australia and later with the warming phase coupled with a brief resurgence of the rainforests which continued until ca. 3 MYA. The earlier drying phase has possibly given rise to the *Similia* and *Avicularia*-

Fig. 9 Lineages-through time plot (LTT) for the subgenus *Archaeosolanum* calculated across age estimates obtained for the tMRCA of the group with BEAST. Blue graphs represent upper and lower 95% HPDs. Time scale ages are shown in millions of years (MY) before present. The plot shows a steady rate of diversification, without significant changes or shifts

Laciniata clades ca. 6.5 MYA, while the later short rain-forest resurgence led to speciation within these two lineages. Biogeographic analyses (DIVA, WAAA) together with molecular clock results indicate that the two major subclades arose and radiated during the Miocene in the period when the Australian environment underwent notable aridification that significantly affected other plant groups (e.g. tribes of Fabaceae) and the environmental changes caused expansion or contraction of their ranges [104]. The two lineages within *Archaeosolanum* were obviously separated due to the fragmentation of their once wider habitat by the growing interior of arid deserts in the Australian landscape. This assumption is clearly supported by all biogeographical analysis carried out here. The *Similia* clade restricted to the SE regions, while the range of *Avicularia-Laciniata* clade to the SW region, as the climate became progressively drier during the Miocene age. Taxa that required wetter climates were restricted to refugia in the highlands or to small favourable habitats (*Avicularia-Laciniata* clade), while others adapted to more arid conditions (*Similia* clade). In the case of *Solanum symonii*, the distribution area became highly restricted to the SE-SW Australian coastlines suggesting a strong maritime shoreline influence. The same pattern is also encountered in the disjunctive present day distribution of the *Similia* clade in Western and Southern Australia, where a substantial gap is preserved within the populations of *S. simile* and *S. capsiciforme*, while this gap is much smaller in the case of *S. symonii* (see Fig. 2). No specimens belonging to the *Avicularia-Laciniata* clade have been collected in Western Australia or just some sporadic records are known.

Diversification in Papua New Guinea

The *trnT-F* data together with the DIVA and WAAA reconstructions supports the hypothesis of an early Australian and New Guinean origin of kangaroo apples followed by a habitat fragmentation and diversification in the eastern region reaching Tasmania and New Zealand. The uplands of New Guinea are very young, originating from a recent vertical uplift along the axis of the island [105]. New Guinea was clearly part of the Australian region as exemplified by its floristic affinities. The extensive ever-wet uplands of the present day did not exist in its current form through most of the period of angiosperm history of the region [106]. *Solanum multivenosum* presumably diversified through vicariance during the continuous uplift of New Guinea and evolved in isolation from other species of the group, adapting to tropical climatic conditions. This could be associated with the recent vertical uplift of the Central Range orogeny of New Guinea, which developed from several geological arcs during the Late Miocene to the Holocene [107]. This orogenesis formed a long

mountainous backbone (ca. 1,300 km) with some peaks over 5,000 m [108]. These mountain ranges presumably promoted speciation by acting as physical barriers and creating a hot, wet climate associated with annual rainfall of over 2,500 mm. This process is analogous to the uplift of the Andes in South America—the centre of genetic diversity for many core groups of *Solanum* lineages—which also resulted in well known important speciations within the genus (see [11]). Therefore other undiscovered species and/or intra- and infraspecific taxa may exist in the high altitude ranges of New Guinea where the subgenus *Archaeosolanum* is poorly known. *Solanum aviculare*, the species nowadays also known to be present in New Guinea, probably originates from a fairly recent colonization, possibly from the Northern Queensland refugia as indicated by DIVA and WAAA.

Future directions

Kangaroo apples represent an early radiation in the evolutionary history of *Solanum*, possibly dating back to the late Miocene. This dispersal—one of the most ancient in the genus—led to the formation of a unique isolated group which split into two separate lineages during the continental aridification of Australia. Despite of the robust phylogenetic hypothesis presented here, many questions regarding the evolutionary history of kangaroo apples remain open. In future studies higher level relatives of this group should be determined by increasing sampling of the poorly characterized African non-spiny group to define its taxonomic limits, and to identify the precise origin of the *Archaeosolanum* clade. In general, the natural variation shown by the group should be explored in order to settle the taxonomy. It is also possible that further undescribed species exist, especially at the high altitudes in the New Guinean mountains where kangaroo apples are still poorly collected. Further field collections are also needed to reveal population genetic processes (e.g. population dynamics, gene flow), especially in New Zealand where the white flowered and ovoid leaf varieties of *S. aviculare* occur. This would give us a more precise view of speciation in this isolated group as well as genetic diversity within and among populations. Such a research programme has already been initiated, and the results are in preparation (G. Weavers, personal comm.). The unique aneuploid chromosome structure—the most interesting feature of this group, indeed the whole genus—should be explored. Some unusual chromosome numbers are also known in other lineages of *Solanum*, but kangaroo apples represent a biologically poorly known case which deserves more attention. Further molecular studies based on biparentally inherited nuclear genes are tools that could be used to

investigate possible hybridization and reticulate evolutionary process of polyploidy within members of the group, as well as to test the obtained hypothesis of phylogenetic relationships.

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