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# Australasian orchid biogeography at continental scale: Molecular phylogenetic insights from the Sun Orchids (*Thelymitra*, Orchidaceae)

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ABSTRACT

Australia harbours a rich and highly endemic orchid flora, with c. 90% of species endemic to the country. Despite that, the biogeographic history of Australasian orchid lineages is only poorly understood. Here we examined evolutionary relationships and the spatio-temporal evolution of the sun orchids (*Thelymitra*, 119 species), which display disjunct distribution patterns frequently found in Australasian orchid lineages. Phylogenetic analyses were conducted based on one nuclear (ITS) and three plastid markers (*matK*, *psbJ-petA*, *ycf1*) using Maximum Likelihood and Bayesian inference. Divergence time estimations were carried out with a relaxed molecular clock in a Bayesian framework. Ancestral ranges were estimated using the dispersal-extinction-cladogenesis model and an area coding based on major disjunctions. The phylogenetic analyses clarified intergeneric relationships within Thelymitrinae, with *Epiblema* being sister to *Thelymitra* plus *Calochilus*, both of which were well-supported. Within *Thelymitra*, eight major and several minor clades were retrieved in the nuclear and plastid phylogenetic reconstructions. Five major clades corresponded to species complexes previously recognized based on morphological characters, whereas other previously recognized species groups were found to be paraphyletic. Conflicting signals between the nuclear and plastid phylogenetic reconstructions provided support for hybridization and plastid capture events both in the deeper evolutionary history of the genus and more recently. Divergence time estimation placed the origin of *Thelymitra* in the late Miocene (c. 10.8 Ma) and the origin of the majority of the main clades within *Thelymitra* during the late Pliocene and early Pleistocene, with the majority of extant species arising during the Pleistocene. Ancestral range reconstruction revealed that the early diversification of the genus in the late Miocene and Pliocene took place predominantly in southwest Australia, where most species with highly restricted distributional ranges occur. Several long-distance dispersal events eastwards across the Nullarbor Plain were inferred, recurrently resulting in lineage divergence within the genus. The predominant eastwards direction of long-distance dispersal events in *Thelymitra* highlights the importance of the prevailing westerly winds in the Southern Hemisphere for the present-day distribution of the genus, giving rise to the *Thelymitra* floras of Tasmania, New Zealand and New Caledonia, which were inferred to be of comparatively recent origin.

## 1. Introduction

Australia harbours a rich and highly endemic orchid flora with over 1300 species, around 90% of which occur nowhere else (Govaerts et al., 2016). Several orchid lineages underwent extensive diversification on the continent, in particular in the terrestrial tribe Diurideae (Orchidoideae), which comprises the majority of Australia's orchid species (Clements, 1989; Govaerts et al., 2016). Diurideae are highly morphologically diverse and a characteristic element of the Australasian orchid flora, displaying their greatest diversity in Australia, and extending to New Zealand, New Guinea, New Caledonia, tropical Asia, as well as the Pacific region (Pridgeon et al., 2001a,b). A family-level phylogenomic study on the historical biogeography of Orchidaceae identified Australia as the second most important source area for migration in the evolutionary history of orchids (Givnish et al., 2016). However, at generic level the spatio-temporal evolution of Australasian

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**Table 1**
Plant material used in this study and GenBank accession numbers of DNA sequences generated for this study. Columns 'Divergence dating nc' and 'Divergence dating pt' show samples included in nuclear and plastid divergence dating analysis; accessions with identical sequences that were represented by one accession in the analysis are indicated by same letter. AD: State Herbarium of South Australia, BRI: Queensland Herbarium, CANB: Australian National Herbarium, CHR: Allan Herbarium, CNS: Australian Tropical Herbarium, MEL: National Herbarium of Victoria.

| Species | Voucher details | DNA extract no. | *mat*K | *psbJ-pet*A | *ycf*1 | ITS | Divergence dating nc | Divergence dating pt |
|---|---|---|---|---|---|---|---|---|
| *Thelymitra aemula* Cheeseman | Molloy, B.P.J. 126/99 (CHR) | CNS_G02953 | MG695070 | MG695175 | MG695295 | MG694945 | x | x |
| *Thelymitra aggericola* D.L.Jones | Rubenach, L. JAJ827 (MEL) | CNS_G02455 | MG695071 | MG695176 | MG695296 | MG694946 | J | x |
| *Thelymitra albiflora* Jeanes 1 | Jones, D.L. 18012 (CANB) | CNS_G02981 | MG695072 | MG695177 | MG695297 | MG694947 | I | x |
| *Thelymitra albiflora* Jeanes 2 | Bates, R.J. 60096 (MEL) | CNS_G03033 | – | MG695178 | MG695298 | – | | |
| *Thelymitra alcockiae* Jeanes 1 | Duretto, M. 1362 (MEL) | CNS_G02987 | – | – | MG695299 | MG694948 | | |
| *Thelymitra alcockiae* Jeanes 2 | Bates, R.J. 60098 (MEL) | CNS_G02992 | MG695073 | MG695179 | – | MG694949 | | |
| *Thelymitra alcockiae* Jeanes 3 | Alcock, K. JAJ888 (MEL) | CNS_G03040 | MG695074 | – | MG695300 | MG694950 | x | x |
| *Thelymitra alpicola* Jeanes | Jeanes, J.A. 938 (MEL) | CNS_G03043 | MG695075 | MG695180 | MG695301 | MG694951 | M | x |
| *Thelymitra alpina* Jeanes 1 | Jeanes, J.A. 808 (MEL) | CNS_G02195 | MG695076 | MG695181 | – | MG694952 | J | |
| *Thelymitra alpina* Jeanes 2 | Rouse, D.T. JAJ1283 (MEL) | CNS_G03091 | – | MG695182 | MG695302 | – | | |
| *Thelymitra antennifera* (Lindl.) Hook.f. 1 | Clements, M.A. 11967A (CANB) | CNS_G01359 | – | – | – | MG694953 | | |
| *Thelymitra antennifera* (Lindl.) Hook.f. 2 | Clements, M.A. 11967B (CANB) | CNS_G01372 | – | – | – | MG694954 | | |
| *Thelymitra antennifera* (Lindl.) Hook.f. 3 | Clements, M.A. 10408 (CANB) | CNS_G02901 | MG695077 | MG695183 | MG695303 | MG694955 | x | x |
| *Thelymitra arenaria* Lindl. | Alcock, K. JAJ889 (MEL) | CNS_G03092 | – | MG695184 | MG695304 | MG694956 | I | x |
| *Thelymitra aristata* Lindl. | Clements, M.A. 11690 (CANB) | CNS_G02890 | MG695078 | MG695185 | MG695305 | MG694957 | x | x |
| *Thelymitra benthamiana* Rchb.f. 1 | Jeanes, J.A. 1090 (MEL) | CNS_G02194 | MG695079 | MG695186 | MG695306 | MG694958 | | |
| *Thelymitra benthamiana* Rchb.f. 2 | Clements, M.A. 10680 (CANB) | CNS_G02886 | MG695080 | MG695187 | MG695307 | MG694959 | E | x |
| *Thelymitra benthamiana* Rchb.f. 3 | Clements, M.A. 10410 (CANB) | CNS_G02895 | – | MG695188 | MG695308 | MG694960 | | |
| *Thelymitra bracteata* J.Z.Weber ex J.Jeanes | Jones, D.L. 18011 (CANB) | CNS_G03046 | MG695081 | MG695189 | MG695309 | MG694961 | I | x |
| *Thelymitra campanulata* Lindl. 1 | Clements, M.A. 11976B (CANB) | CNS_G01091 | MG695082 | MG695190 | – | MG694962 | x | |
| *Thelymitra campanulata* Lindl. 2 | Jeanes, J.A. 1157 (MEL) | CNS_G03000 | – | MG695191 | MG695310 | MG694963 | | |
| *Thelymitra canaliculata* R.Br. | Clements, M.A. 10675 (CANB) | CNS_G02909 | MG695083 | MG695192 | MG695311 | MG694964 | B | x |
| *Thelymitra carnea* R.Br. 1 | Molloy, B.P.J. 104/99 (CHR) | CNS_G02885 | MG695084 | MG695193 | MG695312 | MG694965 | | |
| *Thelymitra carnea* R.Br. 2 | Rouse, D.T. JAJ756 (MEL) | CNS_G02982 | MG695085 | MG695194 | MG695313 | MG694966 | A | x |
| *Thelymitra circumsepta* Fitzg. 1 | Molloy, B.P.J. 048/98 (CHR) | CNS_G02947 | – | MG695195 | MG695314 | MG694967 | | |
| *Thelymitra circumsepta* Fitzg. 2 | Wapstra, H. s.n. (CANB) | CNS_G03017 | – | MG695197 | MG695315 | MG694968 | x | x |
| *Thelymitra circumsepta* Fitzg. 3 | Clements, M.A. 11137 (CANB) | CNS_G02976 | – | MG695196 | – | – | | |
| *Thelymitra cornicina* Rchb.f. 1 | Clements, M.A. 11980A (CANB) | CNS_G01350 | MG695086 | MG695198 | MG695316 | MG694969 | | |
| *Thelymitra cornicina* Rchb.f. 2 | Jeanes, J.A. 1163 (MEL) | CNS_G03032 | MG695087 | MG695199 | MG695317 | MG694970 | x | x |
| *Thelymitra crinita* Lindl. 1 | Clements, M.A. 11975A (CANB) | CNS_G01082 | MG695088 | MG695200 | MG695318 | MG694971 | | |
| *Thelymitra crinita* Lindl. 2 | Clements, M.A. 11975B (CANB) | CNS_G01103 | MG695089 | MG695201 | MG695319 | MG694972 | | |
| *Thelymitra crinita* Lindl. 3 | Clements, M.A. 10400 (CANB) | CNS_G02926 | MG695090 | MG695202 | MG695320 | MG694973 | x | x |
| *Thelymitra cucullata* Rupp | French, C.J. 2823 (MEL) | CNS_G02996 | MG695091 | – | MG695321 | MG694974 | x | x |
| *Thelymitra cyanea* (Lindl.) Benth. 1 | Clements, M.A. 11999 (CANB) | CNS_G02402 | MG695093 | MG695203 | MG695323 | MG694975 | x | |
| *Thelymitra cyanea* (Lindl.) Benth. 2 | Jeanes, J.A. 1291 (MEL) | CNS_G02199 | MG695092 | – | MG695322 | – | | |
| *Thelymitra dedmaniarum* R.S.Rogers | French, C.J. 2796 (MEL) | CNS_G03027 | MG695094 | MG695204 | MG695324 | MG695001 | D | x |
| *Thelymitra epipactoides* F.Muell. | Clements, M.A. 12046 (CANB) | CNS_G02437 | MG695095 | MG695205 | MG695325 | MG694976 | C | x |
| *Thelymitra exigua* Jeanes | Jeanes, J.A. 1171 (MEL) | CNS_G03150 | – | MG695206 | MG695326 | MG694977 | I | x |
| *Thelymitra flexuosa* Endl. 1 | Clements, M.A. 10397 (CANB) | CNS_G02888 | MG695096 | MG695207 | MG695327 | MG694978 | | |
| *Thelymitra flexuosa* Endl. 2 | Clements, M.A. 10411 (CANB) | CNS_G02894 | MG695097 | MG695208 | MG695328 | MG694979 | A | x |
| *Thelymitra flexuosa* Endl. 3 | Clements, M.A. 11971B (CANB) | CNS_G01081 | MG695151 | MG695262 | MG695389 | MG695039 | | |
| *Thelymitra flexuosa* Endl. 4 | Clements, M.A. 11971A (CANB) | CNS_G01369 | – | – | – | MG695040 | | |
| *Thelymitra frenchii* Jeanes | French, C.J. 3215 (MEL) | CNS_G03025 | MG695098 | MG695209 | MG695329 | MG694980 | H | x |
| *Thelymitra fuscolutea* R.Br. 1 | Clements, M.A. s2388 (CANB) | CNS_G02898 | MG695099 | MG695210 | MG695330 | MG694981 | E | |
| *Thelymitra fuscolutea* R.Br. 2 | Clements, M.A. 10683 (CANB) | CNS_G02905 | MG695100 | MG695211 | MG695331 | MG694982 | | |
| *Thelymitra fuscolutea* R.Br. 3 | Clements, M.A. 10664 (CANB) | CNS_G02906 | – | – | MG695332 | MG694983 | | |
| *Thelymitra glaucophylla* Jeanes | Bates, R.J. 60131 (AD) | CNS_G03128 | MG695101 | MG695212 | MG695333 | MG694984 | J | x |
| *Thelymitra graminea* Lindl. | Jeanes, J.A. 834 (MEL) | CNS_G03073 | – | MG695213 | – | MG694985 | K | |
| *Thelymitra grandiflora* Fitzg. 1 | Clements, M.A. 11672 (CANB) | CNS_G02900 | MG695102 | MG695214 | MG695334 | MG694986 | | |
| *Thelymitra grandiflora* Fitzg. 2 | Bates, R.J. 60103 (MEL) | CNS_G03008 | MG695103 | MG695215 | MG695335 | MG694987 | x | x |
| *Thelymitra granitora* D.L.Jones et M.A.Clem. 1 | Clements, M.A. 11952 (CANB) | CNS_G01362 | – | – | – | MG694988 | | |
| *Thelymitra granitora* D.L.Jones et M.A.Clem. 2 | Clements, M.A. 10416 (CANB) | CNS_G02897 | MG695104 | MG695216 | MG695336 | MG694989 | | |

(*continued on next page*)

**Table 1** (*continued*)

| Species | Voucher details | DNA extract no. | *mat*K | *psbJ-pet*A | *ycf*1 | ITS | Divergence dating nc | Divergence dating pt |
|---|---|---|---|---|---|---|---|---|
| *Thelymitra granitora* D.L.Jones et M.A.Clem. 3 | Jeanes, J.A. 1165 (MEL) | CNS_G03039 | MG695105 | MG695217 | MG695337 | MG694990 | H | x |
| *Thelymitra gregaria* D.L.Jones et M.A.Clem. | Jeanes, J.A. 1172 (MEL) | CNS_G02456 | – | MG695218 | MG695338 | – | | x |
| *Thelymitra holmesii* Nicholls | Bates, R.J. 60210 (MEL) | CNS_G03095 | – | – | – | MG694991 | x | |
| *Thelymitra imbricata* D.L.Jones et M.A.Clem. | Jeanes, J.A. 945 (MEL) | CNS_G02196 | MG695106 | – | MG695339 | – | | x |
| *Thelymitra inflata* Jeanes | Bates, R.J. 60091 (MEL) | CNS_G03055 | – | MG695219 | – | – | | |
| *Thelymitra ixioides* Sw. | Jeanes, J.A. 893 (MEL) | CNS_G02198 | MG695107 | – | MG695340 | MG694992 | F | x |
| *Thelymitra jacksonii* Hopper & A.P.Br. ex Jeanes | Jackson, W. JAJ983 (MEL) | CNS_G02999 | MG695108 | MG695220 | MG695341 | MG695003 | D | |
| *Thelymitra jonesii* Jeanes | Jeanes, J.A. 1199 (MEL) | CNS_G02997 | MG695109 | MG695221 | MG695342 | MG694993 | B | x |
| *Thelymitra juncifolia* Lindl. 1 | Bates, R.J. 60100 (MEL) | CNS_G02201 | MG695110 | MG695222 | MG695343 | MG694994 | | |
| *Thelymitra juncifolia* Lindl. 2 | Jeanes, J.A. 968 (MEL) | CNS_G02204 | MG695111 | MG695223 | MG695344 | MG694995 | F | x |
| *Thelymitra longifolia* J.R.Forst. & G.Forst. 1 | Molloy, B.P.J. 038/98 (CHR) | CNS_G02915 | – | – | MG695345 | MG694996 | | |
| *Thelymitra longifolia* J.R.Forst. & G.Forst. 2 | Molloy, B.P.J. 098/99 (CHR) | CNS_G02925 | MG695112 | MG695224 | MG695346 | MG694997 | L | x |
| *Thelymitra longiloba* D.L.Jones et M.A.Clem. | Branwhite, B. JAJ1026 (MEL) | CNS_G03010 | MG695113 | MG695225 | MG695347 | – | | |
| *Thelymitra macrophylla* Lindl. 1 | Jeanes, J.A. 862 (MEL) | CNS_G02203 | MG695114 | MG695226 | – | MG694998 | | |
| *Thelymitra macrophylla* Lindl. 2 | Clements, M.A. s2387 (CANB) | CNS_G02919 | MG695115 | MG695227 | MG695348 | MG694999 | x | x |
| *Thelymitra macrophylla* Lindl. 3 | Clements, M.A. 10390 (CANB) | CNS_G02950 | MG695116 | MG695228 | – | MG695000 | | |
| *Thelymitra magnifica* Jeanes | Jeanes, J.A. 841 (MEL) | CNS_G03001 | MG695117 | MG695229 | MG695349 | MG695002 | D | x |
| *Thelymitra malvina* M.A.Clem,. D.L.Jones & Molloy 1 | Clements, M.A. 11694 (CANB) | CNS_G02887 | MG695118 | MG695230 | MG695350 | MG695005 | | |
| *Thelymitra malvina* M.A.Clem., D.L.Jones & Molloy 2 | Clements, M.A. 11421 (CANB) | CNS_G02891 | MG695119 | MG695231 | MG695351 | MG695006 | | |
| *Thelymitra malvina* M.A.Clem., D.L.Jones & Molloy 3 | Jeanes, J.A. 1192 (MEL) | CNS_G03004 | MG695120 | MG695232 | MG695352 | MG695007 | x | x |
| *Thelymitra matthewsii* Cheeseman | Molloy, B.P.J. 189/00 (CHR) | CNS_G02935 | – | – | MG695353 | MG695008 | x | |
| *Thelymitra media* R.Br. | Rouse, D.T. JAJ890 (MEL) | CNS_G02993 | MG695121 | MG695233 | MG695354 | MG695009 | x | x |
| *Thelymitra megacalyptra* Fitzg. 1 | Jones, D.L. 17255 (CANB) | CNS_G02984 | MG695122 | MG695234 | MG695355 | MG695010 | J | x |
| *Thelymitra megacalyptra* Fitzg. 2 | Jeanes, J.A. 1180 (MEL) | CNS_G03048 | MG695123 | MG695235 | – | MG695011 | | |
| *Thelymitra mucida* Fitzg. 1 | Orchid Research Group ORG2856 (CANB) | CNS_G02889 | MG695124 | MG695236 | MG695356 | MG695012 | | |
| *Thelymitra mucida* Fitzg. 2 | Clements, M.A. 10670 (CANB) | CNS_G02907 | – | MG695237 | MG695357 | MG695013 | | |
| *Thelymitra mucida* Fitzg. 3 | Clements, M.A. 10689 (CANB) | CNS_G02908 | MG695125 | MG695238 | MG695358 | MG695014 | x | x |
| *Thelymitra nuda* R.Br. 1 | Clements, M.A. 11891 (CANB) | CNS_G02404 | MG695126 | – | MG695360 | MG695015 | | |
| *Thelymitra nuda* R.Br. 2 | Clements, M.A. 11883 (CANB) | CNS_G02405 | MG695127 | MG695239 | MG695361 | MG695016 | J | x |
| *Thelymitra nuda* R.Br. 3 | Jeanes, J.A. 782 (MEL) | CNS_G02200 | – | – | MG695359 | – | | |
| *Thelymitra paludosa* Jeanes 1 | Jackson, W. JAJ1121 (MEL) | CNS_G02454 | MG695128 | MG695240 | MG695362 | MG695017 | K | x |
| *Thelymitra paludosa* Jeanes 2 | Jeanes, J.A. JAJ852 (MEL) | CNS_G03035 | – | MG695241 | MG695363 | MG695018 | | |
| *Thelymitra pauciflora* R.Br. 1 | Clements, M.A. 12052 (CANB) | CNS_G02432 | MG695129 | – | MG695364 | MG695019 | x | x |
| *Thelymitra pauciflora* R.Br. 2 | Molloy, B.P.J. 007/98 (CHR) | CNS_G02943 | – | MG695242 | MG695365 | – | | |
| *Thelymitra petrophila* Jeanes | Jeanes, J.A. 1158 (MEL) | CNS_G02202 | MG695130 | MG695243 | MG695366 | MG695020 | K | x |
| *Thelymitra psammophila* C.R.P.Andrews | Orchid Research Group ORG6794 (CANB) | CNS_G02403 | MG695131 | – | MG695367 | MG695021 | x | x |
| *Thelymitra pulcherrima* Jeanes | French, C.J. 2947 (CANB) | CNS_G03011 | MG695132 | MG695244 | MG695368 | MG695022 | G | x |
| *Thelymitra queenslandica* Jeanes 1 | Jones, D.L. 18503 (CANB) | CNS_G02197 | MG695133 | – | MG695369 | MG695023 | | |
| *Thelymitra queenslandica* Jeanes 2 | Micheneau, C. 4 (CNS) | CNS_G02428 | MG695134 | MG695245 | MG695370 | MG695024 | x | x |
| *Thelymitra rubra* Fitzg. 1 | Clements, M.A. 11679 (CANB) | CNS_G02903 | MG695135 | MG695246 | MG695371 | MG695025 | | x |
| *Thelymitra rubra* Fitzg. 2 | Jones, D.L. 18010 (CANB) | CNS_G03057 | – | MG695247 | – | – | | |
| *Thelymitra sanscilia* H.S.Irwin 1 | Molloy, B.P.J. 212/00 (CHR) | CNS_G02945 | MG695136 | MG695248 | MG695372 | MG695026 | | |
| *Thelymitra sanscilia* H.S.Irwin 2 | Molloy, B.P.J. 215/00 (CHR) | CNS_G02946 | MG695137 | MG695249 | MG695373 | MG695027 | M | x |
| *Thelymitra sarasiniana* Kraenzl. 1 | Clements, M.A. 11233 (CANB) | CNS_G02956 | MG695138 | MG695250 | MG695374 | MG695028 | | |
| *Thelymitra sarasiniana* Kraenzl. 2 | Clements, M.A. 11230 (CANB) | CNS_G02957 | MG695139 | MG695251 | MG695375 | MG695029 | x | x |
| *Thelymitra silena* D.L.Jones | Wapstra, H. JAJ786 (MEL) | CNS_G03015 | MG695140 | – | MG695376 | MG695030 | | x |
| *Thelymitra* sp. 1 | Clements, M.A. 11279 (CANB) | CNS_G02955 | MG695145 | MG695256 | MG695382 | MG695033 | | x |
| *Thelymitra* sp. 2 | Clements, M.A. 11280 (CANB) | CNS_G02961 | MG695146 | MG695257 | MG695383 | MG695034 | | x |
| *Thelymitra* sp. “comet” | Molloy, B.P.J. 111/99 (CHR) | CNS_G02933 | – | – | MG695377 | – | | |
| *Thelymitra* sp. “darkie” | Molloy, B.P.J. 022/98 (CHR) | CNS_G02932 | MG695141 | MG695252 | MG695378 | – | | x |
| *Thelymitra* sp. “Mt Clarence” | Clements, M.A. s2391 (CANB) | CNS_G02928 | MG695142 | MG695253 | MG695379 | MG695031 | I | x |
| *Thelymitra* sp. “rough leaf” | Molloy, B.P.J. 021/98 (CHR) | CNS_G02929 | MG695143 | MG695254 | MG695380 | – | | |
| *Thelymitra* sp. “Whakapapa” | Molloy, B.P.J. 245/00 (CHR) | CNS_G02930 | MG695144 | MG695255 | MG695381 | MG695032 | L | x |
| *Thelymitra sparsa* D.L.Jones et M.A.Clem. | Jeanes, J.A. 1315 (MEL) | CNS_G03153 | MG695147 | MG695258 | MG695384 | MG695035 | | x |
| *Thelymitra spiralis* (Lindl.) F.Muell. 1 | Hort, F. 1354 (MEL) | CNS_G02193 | MG695148 | MG695259 | MG695385 | MG695036 | G | |

(*continued on next page*)

**Table 1** (*continued*)

| Species | Voucher details | DNA extract no. | *mat*K | *psbJ-pet*A | *ycf*1 | ITS | Divergence dating nc | Divergence dating pt |
|---|---|---|---|---|---|---|---|---|
| *Thelymitra spiralis* (Lindl.) F.Muell. 2 | Hort, F. 1355 (MEL) | CNS_G02989 | – | MG695260 | MG695386 | MG695037 | G | |
| *Thelymitra spiralis* (Lindl.) F.Muell. 3 | French, C.J. 2865 (MEL) | CNS_G03036 | MG695149 | MG695261 | MG695387 | MG695038 | G | x |
| *Thelymitra stellata* Lindl. | French, C.J. 2120 (CANB) | CNS_G02978 | MG695150 | – | MG695388 | MG695004 | D | x |
| *Thelymitra tholiformis* Molloy & Hatch | Molloy, B.P.J. 127/99 (CHR) | CNS_G02920 | – | MG695263 | MG695390 | MG695041 | | x |
| *Thelymitra tigrina* R.Br. 1 | Clements, M.A. 10690 (CANB) | CNS_G02904 | MG695152 | MG695264 | MG695391 | MG695042 | x | x |
| *Thelymitra tigrina* R.Br. 2 | French, C.J. 3284 (MEL) | CNS_G03005 | – | MG695265 | MG695392 | MG695043 | | |
| *Thelymitra uliginosa* Jeanes 1 | French, C.J. 2904 (MEL) | CNS_G02990 | MG695153 | MG695266 | MG695393 | MG695044 | | |
| *Thelymitra uliginosa* Jeanes 2 | French, C.J. 2914 (MEL) | CNS_G03037 | MG695154 | MG695267 | MG695394 | MG695045 | x | x |
| *Thelymitra uliginosa* Jeanes 3 | French, C.J. 2908 (MEL) | CNS_G03066 | – | MG695268 | MG695395 | MG695046 | | |
| *Thelymitra variegata* (Lindl.) F.Muell. | French, C.J. 2919 (MEL) | CNS_G03059 | – | MG695269 | MG695396 | MG695047 | G | x |
| *Thelymitra venosa* R.Br. | Molloy, B.P.J. 058/98 (CHR) | CNS_G02936 | MG695155 | MG695270 | MG695397 | MG695048 | x | x |
| *Thelymitra villosa* Lindl. | Jeanes, J.A. s.n (MEL) | CNS_G03007 | MG695156 | MG695271 | MG695398 | MG695049 | x | x |
| *Thelymitra vulgaris* Jeanes | Clements, M.A. 10699 (CANB) | CNS_G02896 | MG695157 | MG695272 | MG695399 | MG695050 | I | |
| *Thelymitra xanthotricha* Jeanes | Jeanes, J.A. 1149 (MEL) | CNS_G03014 | MG695158 | – | MG695400 | MG695051 | x | |
| Outgroup taxa | | | | | | | | |
| *Acianthus borealis* D.L.Jones | Jensen, R. 2019 (BRI) | CNS_D2030 | MG695052 | MG695159 | MG695273 | MG694927 | x | x |
| *Caladenia arenicola* Hopper et A.P.Br. | Clements, M.A. 11942 (CANB) | CNS_G01337 | – | MG695160 | MG695275 | MG694929 | x | x |
| *Caladenia denticulata* Lindl. | Clements, M.A. 11018 (CANB) | CNS_G04024 | MG695064 | – | MG695286 | MG694934 | x | x |
| *Calochilus campestris* R.Br. | Clements, M.A. 9933 (CANB) | CNS_G02205 | MG695054 | MG695161 | MG695276 | MG694942 | x | x |
| *Calochilus gracillimus* Rupp | Orchid Research Group ORG4400 (CANB) | CNS_G02235 | MG695055 | MG695162 | MG695277 | – | x | |
| *Calochilus paludosus* R.Br. | Clements, M.A. 11442 (CANB) | CNS_G02209 | MG695056 | MG695163 | MG695278 | MG694943 | x | x |
| *Calochilus robertsonii* Benth. | Rouse, D.T. 212 (CANB) | CNS_G02230 | MG695057 | MG695164 | MG695279 | MG694944 | x | x |
| *Coilochilus neocaledonicum* Schltr. | Clements, M.A. 11248 (CANB) | CNS_G02240 | MG695058 | MG695165 | MG695280 | MG694939 | x | x |
| *Corunastylis nuda* (Hook.f.) D.L.Jones et M.A.Clem. | Dowling, W.M. 390 (CANB) | CNS_G02557 | MG695059 | MG695166 | MG695281 | MG694930 | x | x |
| *Corybas unguiculatus* (R.Brm.) Rchb.f. | Clements, M.A. 10825 (CANB) | CNS_G04119 | MG695053 | – | MG695274 | MG694928 | x | x |
| *Cryptostylis ovata* R.Br. | Orchid Research Group ORG6831 (CANB) | CNS_G02833 | MG695060 | MG695167 | MG695282 | MG694931 | x | x |
| *Diuris oporina* D.L.Jones | Gray, B. 8903 (CNS) | CNS_G03311 | MG695061 | MG695168 | MG695283 | MG694938 | x | x |
| *Epiblema grandiflorum* R. Br. | Orchid Research Group 4468 (CANB) | CNS_G02238 | MG695062 | MG695169 | MG695284 | MG694941 | x | x |
| *Epiblema grandiflorum* R. Br. | French, C.J. 3283 (CANB) | CANB S1079 | – | – | – | AF348029 | | |
| *Glossodia major* R.Br. | Clements, M.A. 12129 (CANB) | CNS_G04332 | MG695063 | MG695170 | MG695285 | MG694933 | x | x |
| *Leporella fimbriata* (Lindl.) A.S.George | Clements, M.A. 10452 (CANB) | CNS_G03289 | MG695065 | MG695171 | MG695287 | MG694935 | x | x |
| *Mecopodum striatum* (R.Br.) D.L.Jones et M.A.Clem. | Clements, M.A. 12009 (CANB) | CNS_G02559 | MG695066 | MG695172 | MG695288 | MG694937 | x | x |
| *Megastylis gigas* (Rchb.f.) Schltr. | Clements, M.A. 11177b (CANB) | CNS_G03418 | MG695067 | – | MG695289 | MG694932 | x | x |
| *Microtis arenaria* Lindl. | Molloy, B.P.J. 211/00 (CHR) | CNS_G02548 | MG695068 | MG695173 | MG695290 | MG694936 | x | x |
| *Plumatichilos barbatus* (Lindl.) Szlach. | Clements, M.A. 11966A (CANB) | CNS_G01354 | MG695069 | – | MG695291 | MG694924 | x | x |
| *Prasophyllum canaliculatum* D.L.Jones | Miles, J. 146983 (CANB) | CNS_G02561 | MG695401 | MG695174 | MG695292 | MG694940 | x | x |
| *Urochilus vittatus* (Lindl.) D.L.Jones et M.A.Clem. | Clements, M.A. 11939A (CANB) | CNS_G01116 | – | – | MG695293 | MG694925 | x | x |
| *Vrydagzynea* sp. | Juswara, L. 15 (CANB) | CNS_G01271 | – | – | MG695294 | MG694926 | x | x |

orchids is still poorly understood.

The genus *Thelymitra* (subtribe Thelymitrinae) is one of the five largest genera in Diurideae (Chase et al., 2015) and comprises 119 species of tuberous geophytic herbs (Govaerts et al., 2016; Bates, 2010; Jeanes, 2013), many of which are considered rare or listed as threatened (46 and 36 spp., respectively). Their flowers have a radial symmetric perianth, an unusual feature in orchids, and possess a characteristic hood-like structure (mitra), formed by the fused column wings (Pridgeon et al., 2001a,b). The nectarless flowers are food-deceptive and attract mainly bees for pollination (Edens-Meier et al., 2014). The flowers have a tendency to open in response to daylight, warm temperatures and humidity, hence their common name Sun Orchids. However, several species are autogamous and barely open their flowers (Pridgeon et al., 2001a,b). Like in all orchids, *Thelymitra* seeds are minute and rely on wind-dispersal.

*Thelymitra* exhibits biogeographical patterns frequently found in Australasian orchid lineages. The majority of species occur in southeast Australia, whereas their centre of endemism lies in the global biodiversity hotspot of southwest Australia (Myers et al., 2000). The genus exhibits several disjunct distributions in Australia, i.e., between southwest Australia, eastern Australia, and Tasmania (AVH, 2016). The distribution of *Thelymitra* extends to New Zealand (22 ssp.), New Caledonia (2 ssp.), New Guinea (2 ssp.), East Timor (1 ssp.), Indonesia (1 ssp.), and the Philippines (1 ssp.) (AVH, 2016; Govaerts et al., 2016). The genus occurs in a wide range of mesic habitats with seasonal climates, from coastal scrub to heathlands and swamps, sclerophyll forests and woodlands to subalpine meadows and alpine herb fields (Pridgeon et al., 2001a,b).

The complex patterns of morphological variation in *Thelymitra*, in particular of floral traits, and the frequent occurrence of natural hybrids have greatly hampered our understanding of evolutionary relationships within the genus. Previous infrageneric classifications were mainly based on column morphology (Bentham and von Mueller, 1873; Lindley, 1840; Pfitzer, 1889) or flower colour (Brown, 1810). Traditionally, three sections are discerned within the genus (*Cucullaria, Macdonaldia*, and *Biaurella*); but the infrageneric classification has been regarded as not comprehensive and in need of revision (Pridgeon et al., 2001a,b). Jones (2006) recognised 16 informal groups within *Thelymitra*, mainly defined by column morphology and flower colour. Six Australian species complexes within *Thelymitra* have been taxonomically revised (Jeanes, 2001, 2004, 2006, 2009, 2010, 2011, 2013). However, no molecular phylogenetic studies in *Thelymitra* are available to assess whether previous infrageneric classifications adequately reflect evolutionary relationships.

Previous molecular phylogenetic studies supported the placement of *Thelymitra* in the tribe Diurideae (Cameron et al., 1999; Clements et al., 2002; Givnish et al., 2015; Kores et al., 2001). The monophyly of subtribe Thelymitrinae including *Thelymitra*, *Calochilus*, and the monotypic genus *Epiblema* was confirmed more recently (Weston et al., 2014). However, intergeneric relationships remained unclear due to conflicting results between nuclear and plastid phylogenetic reconstructions (Weston et al., 2014). Phylogenetic relationships based on the Internal Transcribed Spacer (ITS) found that *Calochilus* is sister to a clade in which *Epiblema* was nested within *Thelymitra* (Clements et al., 2002; Weston et al. 2014). On the other hand, a study based on several plastid markers recovered *Epiblema* as sister to a clade comprising *Thelymitra* and *Calochilus* (Weston et al., 2014). Both studies focussed on broad-level phylogenetic relationships in Diurideae, and thus sampling in Thelymitrinae was limited. So far, molecular phylogenetic studies elucidating infrageneric relationships in *Thelymitra* and the spatio-temporal evolution of the genus are still lacking.

This study aimed to use nuclear and plastid markers to (a) clarify intergeneric relationships in Thelymitrinae, (b) infer phylogenetic relationships within *Thelymitra*, (c) estimate divergence times within the genus, and (d) reconstruct the biogeographical history of *Thelymitra*.

## 2. Material and methods

### 2.1. Taxon sampling

In total, 143 orchid samples were included in the study. For *Thelymitra,* 121 samples were included, representing 69 species and five as yet undescribed taxa. Four *Calochilus* species and the monospecific genus *Epiblema* were sampled to represent the remaining two genera within subtribe Thelymitrinae. As outgroups, representatives of seven subtribes of Diurideae (Acianthinae, Caladeniinae, Cryptostylidinae, Diuridinae, Megastylidinae, Prasophyllinae) were sampled with one to four species each, and tribe Cranichideae, sister group to Diurideae, was represented with three samples. Voucher details and GenBank accession numbers are provided in Table 1.

### 2.2. DNA extraction, amplification, sequencing, and alignment

Total genomic DNA was extracted from lyophilized leaf and/or stem tissue using commercial DNA extraction kits (DNeasy 96 plant kit and DNeasy plant mini kit, Qiagen, Hilden, Germany) following the manufacturer's protocols. In total, four markers were amplified and sequenced, the nuclear internal transcribed spacer region (ITS), the plastid genes *matK* and *ycf1*, and the intergenic spacer *psbJ-petA*. The following primer pairs were used for amplification: primers 17SE and 26SE for the ITS region (Sun et al., 1994), primers 19F (Molvray et al., 2000) and 1326R for *matK* (Cuénoud et al., 2002), primers 3720F and intR for *ycf1* (Neubig et al., 2009), and primers *psbJ* and *petA* for *psbJ-petA* (Shaw et al., 2007).

For the plastid markers, each PCR reaction contained 3 µl template DNA [2–10 ng/µl], 0.5 µl of each primer [10 µM], 2 µl 10x PCR buffer including 1.5 mM $MgCl_2$ (Kapa Biosystems, Wilmington, MA, USA), 0.5 µl $MgCl_2$ [25 mM], 0.9 µl bovine serum albumin [4 mg/ml], 0.5 µl deoxynucleotide triphosphates [10 mM] (Kapa Biosystems), 0.2 µl *Taq* polymerase [5 units/10 µl] (Kapa Biosystems), and 12.9 µl ultrapure water (Millipore, Merck, Bayeswater, Australia). For ITS, 1 µl of 100% dimethyl sulfoxide was added to the PCR mix, adjusting the volume of ultrapure water to 11.9 µl. For amplification of ITS, a touchdown protocol was used with an initial denaturation at 94 °C for 2 min followed by seven cycles with denaturation at 94 °C for 45 s, annealing at 66 °C to 60 °C for 1 min (incrementally reduced by 1 °C per cycle) and elongation at 72 °C for 45 s, followed by 28 cycles with an annealing temperature at 49 °C for 1 min, and a final elongation at 72 °C for 5 min. For amplification of *ycf1*, an initial denaturation was carried out at 94 °C for 3 min followed by ten cycles of denaturation at 94 °C for 30 s, annealing at 60 °C to 51 °C for 60 s (incrementally reduced by 1 °C per cycle) and elongation at 72 °C for 3 min, followed by 30 cycles with an annealing temperature of 50 °C, and a final elongation at 72 °C for 5 min. Thermocycling conditions for the *matK* and *psbJ-petA* started with an initial denaturation of 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 49 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 5 min. Thermocycling was carried out in a Realplex2 Mastercycler (Eppendorf, Hamburg, Germany). PCR products were cleaned using 7.5 µl of PCR product, 0.25 µl thermosensitive alkaline exonuclease [1 unit/µl] (Thermo Scientific, Waltham, USA), 1 µl FastAP buffer (Thermo Scientific) and 1.25 µl ultrapure water (Millipore) and an incubation for 15 min at 37 °C, followed by 15 min at 85 °C. Sequencing reactions were carried out using the BigDye Terminator kit 3.1 (Thermo Scientific) following the manufacturer's protocol and sequencing runs were conducted on an AB3730*xl* sequencer (Thermo Scientific) at the Australian Genome Research Facility, Melbourne, Australia.

Raw sequences for each marker were assembled and edited using Geneious R9 (Kearse et al., 2012). Four ITS sequences showed ambiguous base calls consistent with the presence of multiple ITS copies and therefore were excluded from subsequent analyses (*Thelymitra rubra* 2, *T.* sp. "darkie", *T.* sp. "rough leaf", and *T. luteocilium*). The four loci

were aligned separately using the MAFFT plugin for Geneious 1.3.3 (Katoh and Standley, 2013), and alignments were subsequently manually edited in Geneious. DNA sequences generated for this study were deposited on GenBank (Table 1).

### 2.3. Phylogenetic analyses

For the phylogenetic analyses, the best-fit substitution model was selected for each marker using jModeltest 2.1.7 (Darriba et al., 2012) based on the Akaike Information Criterion AIC (Akaike, 1974). Maximum likelihood (ML) analyses were conducted in RAxML 8.1.2 (Stamatakis, 2014) with the rapid bootstrap option in effect and 1000 bootstrap replicates. Bayesian analyses were carried out in MrBayes 3.2.5 (Ronquist and Huelsenbeck, 2003), with two independent runs and three heated chains with two million generations to ensure the standard deviation of split frequencies were below 0.01. Trees were sampled every 4000th generation and a consensus tree including all compatible groups was created after removing a burn-in of 20%. All analyses were performed with the GTR + G substitution model, which was the best-fit model available in the software packages used (RAxML and MrBayes). Phylogenetic analyses were first carried out for each marker separately and resulting trees were visually examined for incongruences. Congruence of the trees based on the plastid markers (*matK*, *ycf1*, and *psbJ-petA*) allowed concatenation of all plastid markers for combined analysis. The combined analysis was performed under the same configurations as for the separate markers with the only exception that the Bayesian inference was run with five million generations. Several incongruences were detected between the inferred phylogenies based on the plastid and the nuclear markers, which received moderate to high statistical support. Thus, the plastid and nuclear markers were not subjected to a combined analysis.

### 2.4. Divergence time estimation

Bayesian divergence time estimation was carried out in BEAST 2.4.2 (Bouckaert et al., 2014; Drummond and Rambaut, 2007). A secondary calibration approach was used due to the absence of fossil records in Diurideae. Estimated node ages were taken from Chomicki et al. (2015), based on an uncorrelated lognormal relaxed clock with one maximum constraint (normal distribution) and three internal fossil constraints (hard bound, gamma distributions) for the family-wide divergence time estimation in Orchidaceae. In our analysis, secondary calibrations were set as normally distributed priors for two nodes: the Diurideae stem node (offset = 56.2 Ma, SD = 6.4) and the Diurideae crown node (offset = 49.7 Ma, SD = 5.8). To reduce biases in the divergence time estimations that can result from uneven sampling between the in- and outgroup (Linder et al., 2005; Muellner et al., 2016), two consecutive nodes were used for the secondary calibration.

BEAST analyses were carried out for the nuclear data set (ITS) and the plastid dataset (*matK*, *ycf1*, and *psbJ-petA*) applying a strict clock model and a relaxed clock model. The datasets were optimized for divergence dating to contain divergent sequences only and one accession per species. In cases where several species had identical sequences, these were represented by only one of the species in the analysis. In the chronogram, all omitted species were added to the representative species as part of a polytomy with zero branch length. The final matrices contained 39 *Thelymitra* species for the nuclear and 69 for the plastid dataset (see Table 1). In order to conserve the backbone tree topology according to a previous molecular phylogenetic study in Diurideae (Kores et al., 2001), we enforced the monophyly of two groups in the analysis: (1) the Diurideae, (2) a clade within Diurideae formed by *Calochilus*, *Coilochilus*, *Cryptostylis*, *Diuris*, *Epiblema*, *Leporella*, *Megastylis*, and *Thelymitra*. We used the GTR + G substitution model with empirical base frequencies, a Yule tree prior, and the strict clock model as well as the uncorrelated relaxed clock model with rates drawn from a lognormal distribution. For both datasets, four analyses were run for the strict and eight for the relaxed clock model, each with 11 million generations. For each clock model, one analysis with an empty alignment was carried out to assess the influence of the chosen priors on the resulting posteriors. The convergence of runs was assessed in Tracer 1.6 (estimated sample sizes > 200) and the burn-in phase determined. Multiple runs were combined in LogCombiner (BEAST 2.4.2 package) discarding a burn-in of 10% and resampling set to obtain approximately 10,000 trees. Finally, TreeAnnotator (Drummond and Rambaut, 2007) was used to create annotated maximum clade credibility trees with mean node heights.

### 2.5. Ancestral range estimation

The biogeographical history of *Thelymitra* was inferred via ancestral range estimation using the R package BioGeoBEARS (Matzke, 2013). Two models of biogeographical range inheritance were compared: a maximum likelihood version of dispersal-vicariance (ML-DIVA, Ronquist, 1997; Matzke, 2014) and dispersal-extinction-cladogenesis (DEC, Ree and Smith, 2008). BioGeoBEARS implements one additional range evolution scenario, the founder event, in which one descendant reaches a new area different from the ancestral area (Matzke, 2014). This parameter (J) is added to the existing models. All four models (DEC, DEC + J, ML-DIVA, ML-DIVA + J) were run on the maximum clade credibility tree based on the relaxed molecular clock analysis on the nuclear dataset and the resulting likelihood values were compared using the Akaike Information Criterion (Akaike, 1974) to determine best model fit.

For the ancestral area analysis, 11,084 distribution records for *Thelymitra* were downloaded from the Australian Virtual Herbarium (AVH, 2016) and used to assess the distribution of the genus. Five biogeographic areas were coded based on major disjunctions in the distribution of *Thelymitra*: southwest Australia (W), eastern Australia (E), Tasmania (T), New Zealand (Z), and New Caledonia (C). Species from New Guinea, Indonesia or the Philippines (*T. forbesii, T. javanica, T. papuana*) were not available for the study, and thus these distributions were not included in the coding.

Ancestral range estimations were carried out based on the BEAST chronogram of the nuclear ITS region only. Due to patterns of past hybridization and subsequent plastid capture retrieved in the phylogenetic analyses, we refrained from carrying out ancestral range analyses based on the plastid data set. During the optimization of the dataset for divergence dating, taxa with identical sequences were removed. In cases where the biogeographical coding of a species did not comprise the distribution of the other species represented by the terminal taxon, ancestral areas were combined for the species the terminal taxon represented, e.g., the two species with identical sequences but different distributions *T. canaliculata* (W) and *T. jonesii* (T) were represented by a terminal node coded (WT). The maximum number of combined areas was set to four based on the highest number of combined areas in the sampling. Area combinations and dispersal probabilities were left unconstrained.

## 3. Results

### 3.1. Phylogenetic analyses

Sequencing of one nuclear and three plastid markers resulted in alignments with 1,337 base pairs (bp) and 109 accessions (*matK*), 1502 bp and 114 accessions (*psbJ-petA*), 799 bp and 126 accessions (*ycf1*), and 789 bp and 128 accessions (ITS). The combined plastid matrix contained 3,587 bp and 137 accessions (Table 1). Alignments and resulting trees were uploaded to TreeBase (submission no. 22660).

Maximum likelihood and Bayesian phylogenetic inference based on both the combined plastid and nuclear dataset retrieved Thelymitrinae as well-supported (bootstrap percentages for the plastid dataset: $BS_{pt}$ 90, bootstrap percentages for the nuclear data set: $BS_{nc}$ 97) with

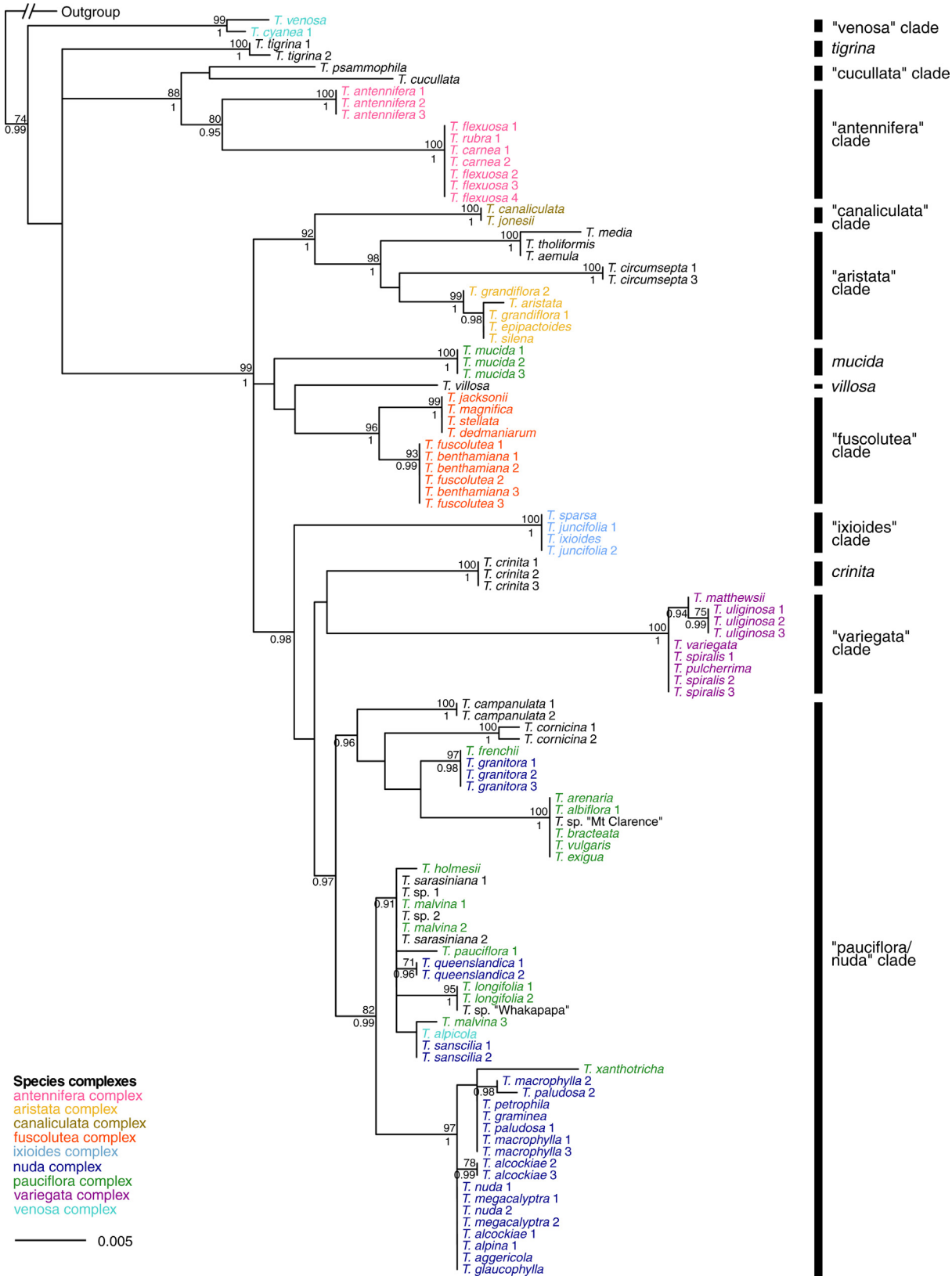


**Fig. 1.** Phylogenetic relationships in *Thelymitra* inferred via Maximum Likelihood analysis of the nuclear ITS region. Bootstrap percentages are given above branches; posterior probabilities from a corresponding Bayesian inference are reported below branches. Outgroup relationships are provided in Suppl. Fig. S1a.

*Epiblema* in sister group position to *Thelymitra* and *Calochilus* (Suppl. Fig. S1). The latter two were resolved as sister groups to each other with high support. The monophyly of *Calochilus* was highly supported in all analyses and the monophyly of *Thelymitra* received high support in the plastid phylogenetic reconstructions ($BS_{pt}$ 100, posterior probabilities for the plastid dataset $PP_{pt}$ 0.99) and low to high support in the nuclear phylogenetic inferences ($BS_{nc}$ 74, posterior probabilities for the nuclear data set $PP_{nc}$ 0.99) (Suppl. Fig. S1).

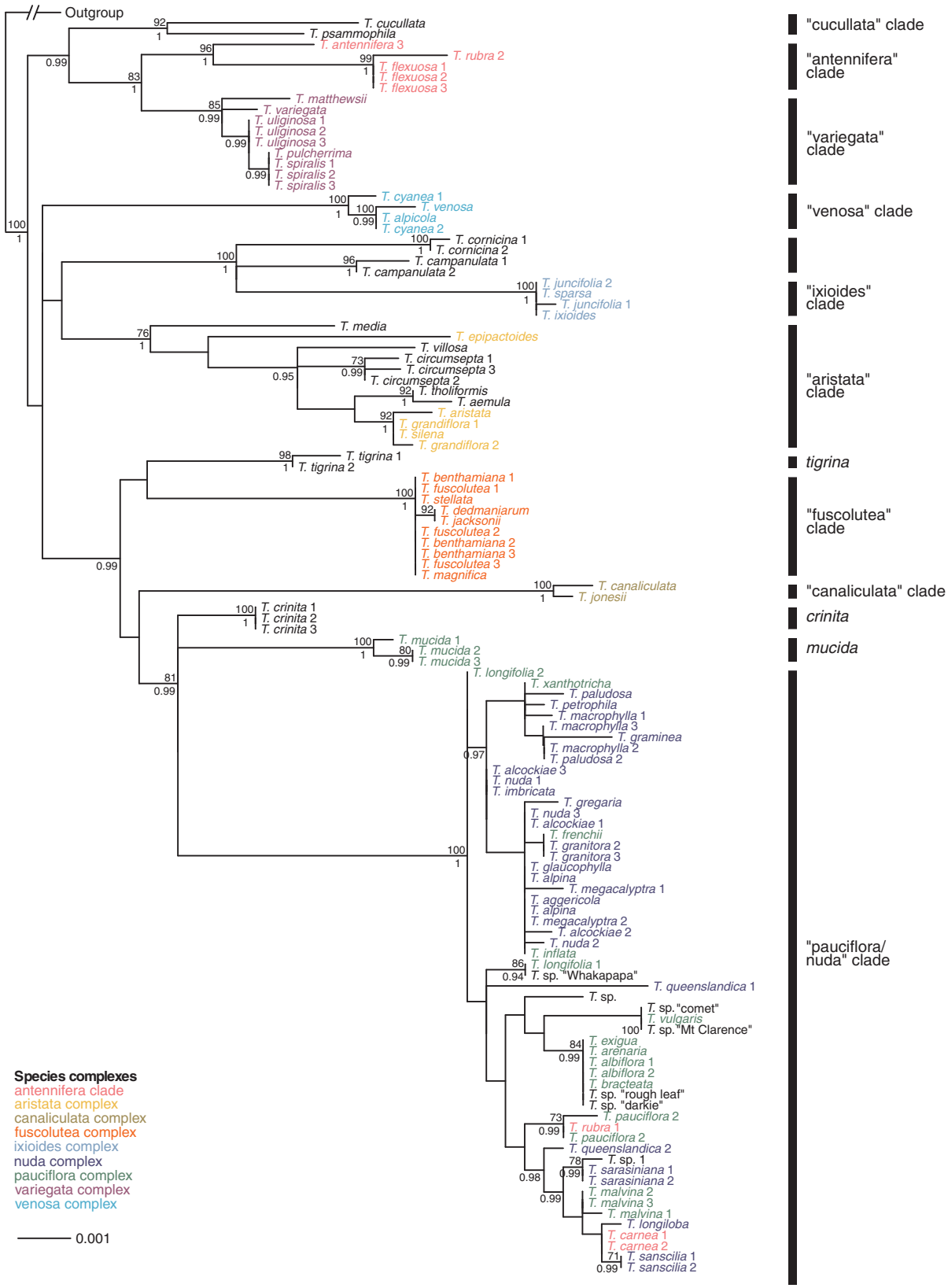


**Fig. 2.** Phylogenetic relationships in *Thelymitra* inferred via Maximum Likelihood analysis of three plastid markers (*matK*, *ycf1*, *psbJ-petA*). Bootstrap percentages are given above branches; posterior probabilities from a corresponding Bayesian inference are reported below branches. Outgroup relationships are provided in Suppl. Fig. S1b.

Within *Thelymitra*, eight main clades were retrieved in the nuclear and plastid phylogenetic reconstructions, as well as several clades comprising a single species each (Fig. 1, Fig. 2). The "variegata" clade comprised all sampled species of the "variegata" complex *sensu* Jeanes (2013) (*T. matthewsii, T. pulcherrima, T. spiralis, T. uliginosa, T. variegata*) and received moderate to high support ($BS_{pt}$ 85, $PP_{pt}$ 0.99, $BS_{nc}$ 100,

$PP_{nc}$ 1). The "fuscolutea" clade encompassed all representatives of the fuscolutea complex *sensu* Jeanes (2006) (*T. benthamiana*, *T. dedmaniarum*, *T. fuscolutea*, *T. jacksonii*, *T. magnifica*, *T. stellata*) and was highly supported ($BS_{pt}$ 100, $PP_{pt}$ 1, $BS_{nc}$ 96, $PP_{nc}$ 1). Relationships within the complex remained unresolved in the plastid trees, whereas in the nuclear trees two highly supported subclades were retrieved: one with *T. dedmaniarum, T. jacksonii*, *T. magnifica*, *T. stellata,* sister to a clade formed by *T. benthamiana* and *T. fuscolutea* (Fig. 2). The "venosa" clade contained three representatives of the venosa complex *sensu* Jeanes (2012) (*T. alpicola*, *T. cyanea*, *T. vericosa*) and received high support in all analyses ($BS_{pt}$ 100, $PP_{pt}$ 1; $BS_{nc}$ 99, $PP_{nc}$ 1). The "ixioides" clade comprised all sampled species of group 5 *sensu* Jones (2006) (*T. ixioides*, *T. juncifolia*, *T. sparsa*) and obtained maximum support in all analyses. Interspecific relationships in the "ixioides" clade remained unresolved due to a lack of genetic divergence. The "canaliculata" clade was formed by the two representatives of the canaliculata complex *sensu* Jeanes (2001) (*T. canaliculata*, *T. jonesii*) and received maximum support in all analyses. The "antennifera" clade obtained moderate support values in the nuclear trees ($BS_{nc}$ 80, $PP_{nc}$ 0.97) and comprised all representatives of group 10 *sensu* Jones (2006) (*T. antennifera, T. flexuosa, T. rubra, T. carnea*). *Thelymitra antennifera* was retrieved in sister group position to the remaining taxa of this clade with moderate support values ($BS_{nc}$ 80, $PP_{nc}$ 0.97). In the plastid trees, *T. antennifera, T. flexuosa,* and one accession of *T. rubra* formed a well-supported clade ($BS_{pt}$ 96, PP 1). Nevertheless, *T. carnea* and one of two accessions of *T. rubra* were nested within the large "pauciflora/nuda" clade, resulting in incongruence between the plastid and nuclear data sets (Fig. 1, Fig. 2).

The "aristata" clade received low to high support values in plastid and nuclear trees ($BS_{pt}$ 76, $PP_{pt}$ 1; $BS_{nc}$ 92, $PP_{nc}$ 1) and contained all representatives of the aristata complex *sensu* Jeanes (2011) plus several other species that have not been assigned to any of the species complexes previously delimited (*T. media*, *T. circumsepta*, *T. tholiformis*, *T. aemula*) (Fig. 1, Fig. 2). *Thelymitra villosa* grouped within the "aristata" clade in the plastid trees, whereas it was part of a larger clade harbouring *T. mucida* and the "fuscolutea" clade, however the latter relationships remained unsupported (Fig. 1, Fig. 2). The "pauciflora/nuda" clade constituted the largest clade and comprised all representatives of the pauciflora and the nuda complexes *sensu* Jeanes (2004, 2013), with the exception of *T. mucida* from the pauciflora complex, for which the phylogenetic position remained unclear due to a lack of statistical support. Both species complexes were found to be paraphyletic.

Multiple incongruences were found between the plastid and the nuclear phylogenetic reconstructions (Fig. 1, Fig. 2). A major incongruence was found in the position of the "variegata" clade. In the nuclear trees, the "variegata" clade was part of a large clade comprising the "ixioides" clade, the "variegata" clade, and *T. crinita* (PP 0.98). However, in the plastid trees, the "variegata" clade was found in sister group position to the "antennifera" clade, which received moderate to high support (BS 83, PP 1). Another incongruence was found in phylogenetic positioning of *T. rubra* and *T. carnea.* The two species were part of the moderately supported "antennifera" clade in the nuclear phylogeny constructions, whereas they were nested within the "pauciflora/nuda" clade in the plastid trees (Fig. 1, Fig. 2). Further, *T. campanulata* and *T. cornicina* formed part of the "pauciflora/nuda" clade in the nuclear trees, whereas they were found in sister group position to the "ixioides" clade with high support values ($BS_{pt}$ 100, $PP_{pt}$ 1) in the plastid trees (Fig. 1, Fig. 2). The morphological diversity within *Thelymitra* is depicted in Fig. 3 with representative species from each clade.

### 3.2. Divergence time estimates

The four divergence age estimations of the nuclear and plastid dataset, each with a strict and a relaxed clock model, resulted in overall similar age estimates. The chronograms from the analyses using strict clock models displayed smaller 95% highest posterior density intervals (HPD), which in most nodes were included within the larger HDP of the analyses applying a relaxed clock model (Suppl. Figs. S2–S4). The age estimates of the plastid dataset resulted in slightly older estimates of the crown age of *Thelymitra* compared to the nuclear dataset, 7.9 Ma (HDP: 5.5–10.9) versus 6.1 Ma (HDP: 4.4–7.9) with the relaxed and 6.2 Ma (HDP: 4.8–7.6) versus 5.8 Ma (HDP: 4.4–7.3) with the strict clock model. The younger ages of the nuclear dataset were contained in the HPD intervals of the plastid set and represent a more conservative age estimates. We therefore refer in the following to the age estimates based on the relaxed clock of the nuclear dataset.

The divergence age estimation resulted in a stem age for Thelymitrinae of 24.9 Ma (HPD: 18.9–30.9), in the Oligocene. The divergence of *Epiblema* from the lineage that gave rise to *Thelymitra* and *Calochilus* occurred 18.7 Ma (HDP: 14.1–23.8), in the early Miocene. The stem age for *Thelymitra* and for *Calochilus* was estimated to 10.8 Ma (HDP: 7.9–13.9), in the mid Miocene (Fig. 4A).

Crown diversification of *Thelymitra* was inferred to have started 6.1 Ma (HDP: 4.4–7.9), in the late Miocene (Fig. 4B). The crown age of the first diverging lineage in *Thelymitra*, the "venosa" clade, was dated to 0.7 Ma (HDP: 0.1–1.4), in the late Pleistocene. The next diverging lineage comprised *T. tigrina*, the "cucullata" clade, and the "antennifera" clade, and had a stem age of 5.7 Ma (HDP: 4.1–7.3), in the late Miocene. The divergence between *T. tigrina* and the rest of this clade was estimated to have occurred 4.9 Ma (HDP: 3.4–6.6), during the early Pliocene. The "cucullata" and "antennifera" clades diverged from each other 3.2 Ma (HDP: 2–4.6) during the late Pliocene and had crown ages of 2.3 Ma (HDP: 1.2–3.4) and 2.4 Ma (HDP: 1.3–3.5) in the early Pleistocene, respectively.

The crown age of the remainder of the genus was dated to 4.1 Ma (HDP: 3.1–5.3), in the early Pliocene. Within this clade, the "canaliculata" and the "aristata" clades diverged from each other 3.1 Ma (HDP: 2.1–4.2), in the late Pliocene, and the crown diversification of the "aristata" clade commenced 2.3 Ma (HDP: 1.5–3.2), in the early Pleistocene. The stem age of the clade containing *T. mucida*, *T. villosa*, and the "fuscolutea" clade was dated to 3.1 Ma (HDP: 2.1–4.2). Crown diversification of the "fuscolutea" clade was estimated to have commenced 0.9 Ma (HDP: 0.3–1.7), in the late Pleistocene. The stem age of the "ixioides" clade was estimated to 3.5 Ma (HDP: 2.5–4.5) and the divergence of *T. crinita* from the "variegata" clade was dated to 2.8 Ma (HDP: 1.9–3.7), both in the late Pliocene. Crown diversification of the "variegata" clade started 0.4 Ma (HDP: 0.1–0.7), in the late Pleistocene. The stem age of the "pauciflora/nuda" clade was estimated to 3.2 Ma (HDP: 2.3–4.1) and crown diversification started 2.7 Ma (HDP: 1.8–3.5), both in the late Pliocene.

### 3.3. Ancestral range analyses

The comparison of the fit of the nuclear data set to the four biogeographic models, based on the corrected Akaike Information Criterion (AICc), favoured the DEC model (AICc 270.7), closely followed by the DEC model plus founder event (+J) (AICc 271.8). The ML-DIVA model and the ML-DIVA + J model received less favourable AICc scores with 279.3 and 281.5, respectively (Suppl. Table S1). Thus, in the following the results from the ancestral range analysis based on the DEC model are presented.

The ancestral range analysis yielded an ambiguous result for the ancestral range of the most recent common ancestor (MRCA) of *Thelymitra* (Fig. 5, Suppl. Table S2). The ancestral range of the first diverging lineage in *Thelymitra,* the "venosa" clade, was inferred as being widespread, with occurrences in eastern Australia, Tasmania, and New Zealand. This ancestral range scenario received a relative probability (rel. prob.) of 60%. For the MRCA of the remainder of the genus, southwest Australia was inferred as the most likely ancestral range, receiving a rel. prob. of 74%, and dated to the late Miocene. The ancestral range analysis inferred that the ancestral range of *Thelymitra* lineages during the Pliocene was predominantly southwest Australia,


**Fig. 3.** Floral diversity in *Thelymitra*, illustrating the phenotypic diversity within the genus through representatives of the major clades. A. *T. cyanea* ("venosa" clade); B. *T. cucullata* ("cucullata" clade); C. *T. antennifera* ("antennifera" clade); D. *T. canaliculata* ("canaliculata" clade); E. *T. aristata* ("aristata" clade); F. *T. fuscolutea* ("fuscolutea" clade); G. *T. ixioides* ("ixioides" clade); H. *T. variegata* ("variegata" clade); I. *T. nuda* ("pauciflora/nuda" clade). Photos by Lars Nauheimer (A, E, G, I), Mark Clements (B), and Noel Hoffman (C, D, F, H).

such as for the MRCA of the clade that comprised the "cucullata" clade, the "antennifera" clade, and *T. tigrina* (rel. prob. 88%), the MRCA of the clade that contained the "fuscolutea" clade, *T. mucida*, and *T. villosa* (rel. prob. 82%), and the MRCA of the clade that comprised the "pauciflora/nuda" clade, the "variegata" clade, and *T. crinita* (rel. prob. 90%, Fig. 5). Other lineages that arose during the Pliocene and for which southwest Australia was inferred as ancestral range were the "pauciflora/nuda" clade (late Pliocene), the "antennifera" clade (late Pliocene), the "cucullata" clade (late Pliocene), as well as the lineages that gave rise to *T. tigrina* (early Pliocene) and *T. crinita* (late Pliocene).

Several biogeographic events dating back to as early as the Pliocene were inferred: (1) at least three range expansions from southwest Australia eastwards (a) in the MRCA of the "variegata" clade, (b) in the MRCA of one of the two major lineages within the "pauciflora/nuda" clade, and (c) in the lineage that gave rise to *T. mucida*, (2) one range shift from southwest Australia to eastern Australia, Tasmania, and New Zealand in the MRCA of the "ixioides" clade (rel. prob. 63%). The ancestral range for the "aristata" clade remained ambiguous with the highest relative probabilities for eastern Australia (rel. prob. 24%) and for Tasmania (rel. prob. 20%, Fig. 5).

The ancestral range reconstruction showed that the majority of range expansions and range shifts occurred relatively recently, from the Pleistocene onwards (Fig. 5). The ancestral range analysis revealed at least seven independent dispersals from mainland Australia to New

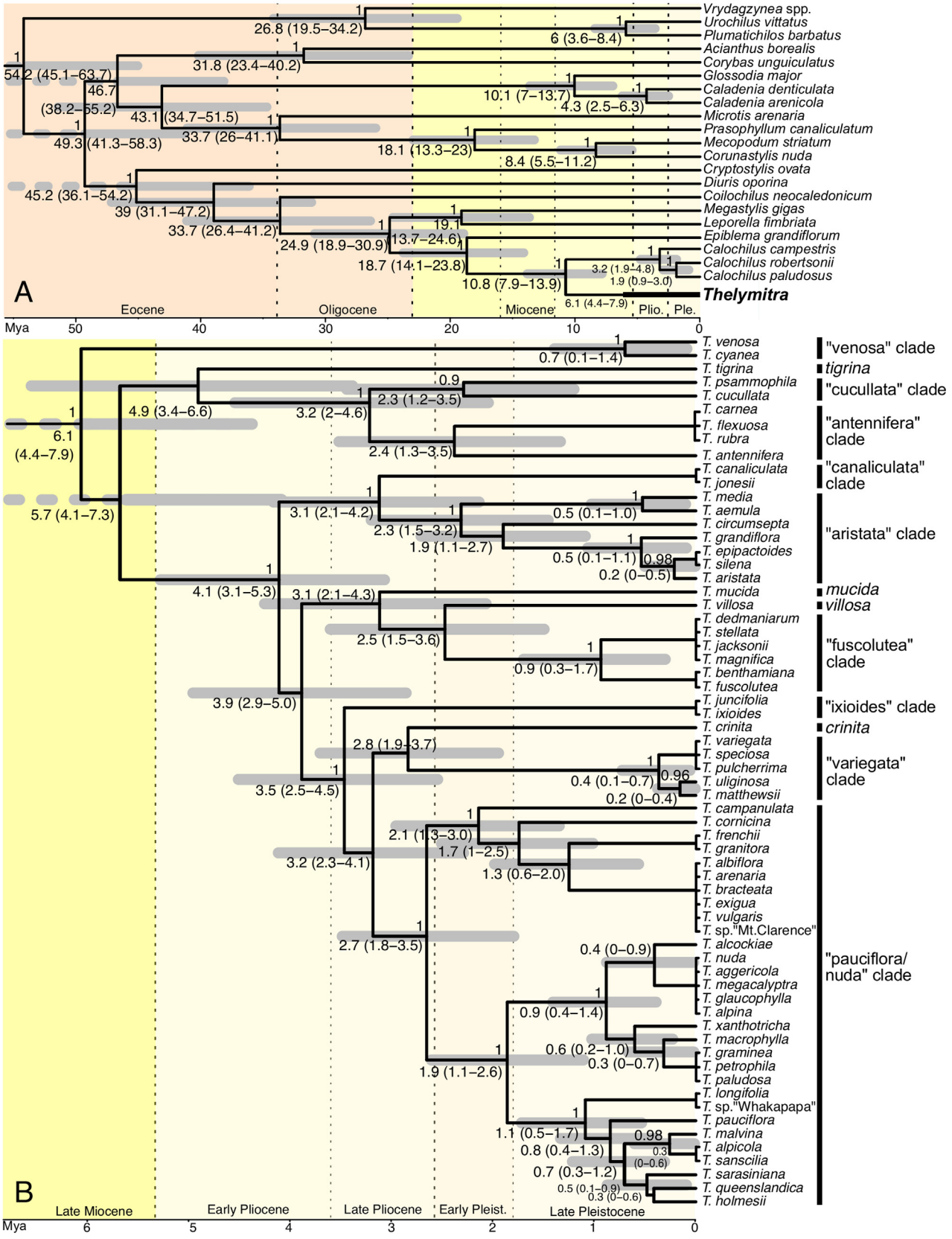


**Fig. 4.** Divergence time estimation for Thelymitrinae based on the nuclear dataset (ITS) under a relaxed clock model. Maximum clade credibility tree with mean node heights showing A) outgroup with *Thelymitra* represented by one lineage (bold for crown group) and B) *Thelymitra*. Posterior probabilities of supported nodes (PP ≥ 0.9) are depicted above branches. Divergence times with their 95% highest posterior density (HDP) ranges are displayed below the branches. HDP intervals are depicted by a grey bar.

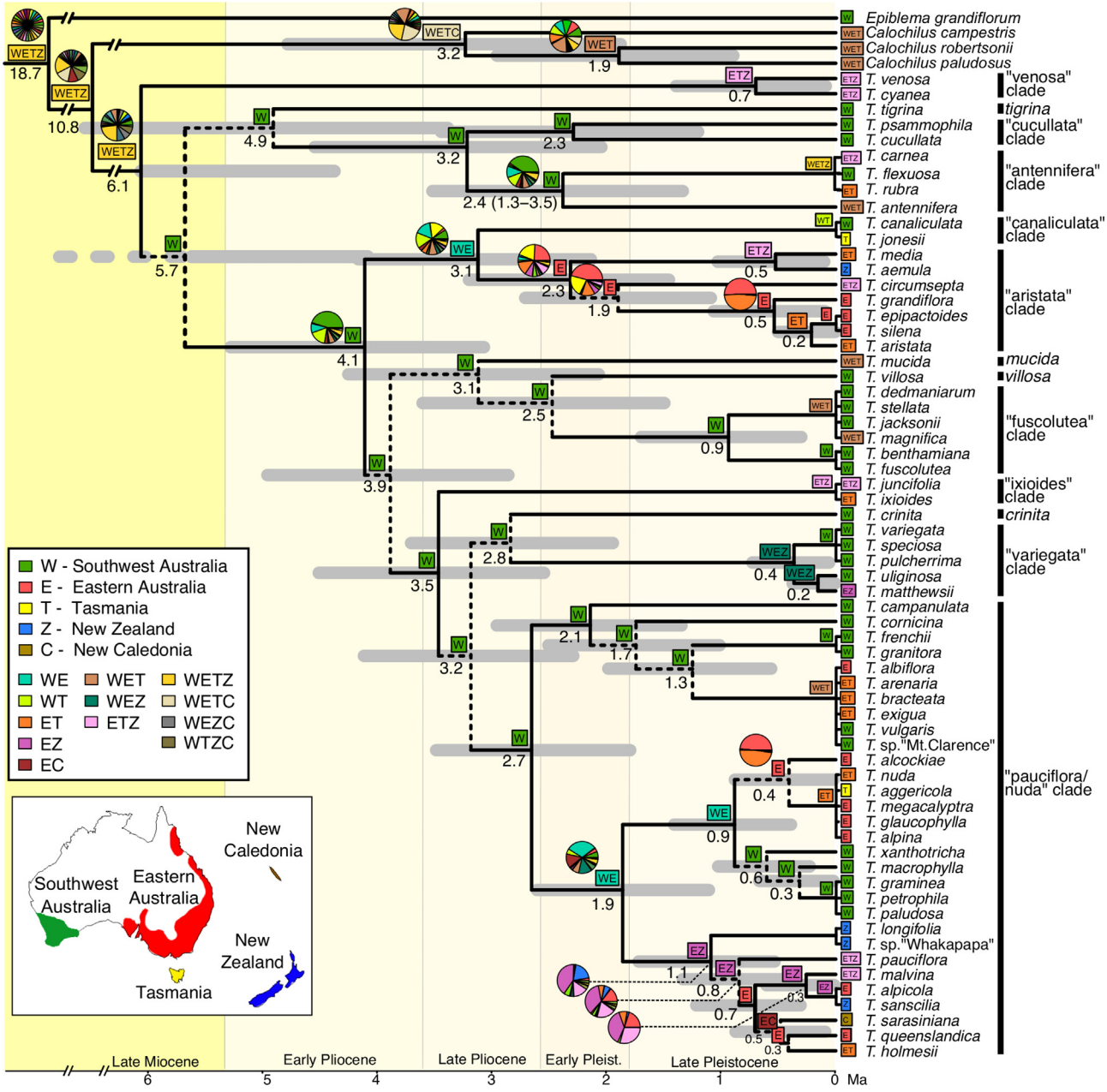


**Fig. 5.** Ancestral range reconstruction for *Thelymitra* based on maximum clade credibility tree obtained for the nuclear dataset (ITS) under a relaxed clock model. Ancestral range estimations with the highest probability are shown in boxes above nodes. For instances in which the most probable area received less than 51%, pie diagrams are displayed showing probabilities of all estimated ranges. Area coding of each accession is shown at tips of the tree. Colour legend and map for area coding are displayed in inserts. Probability values of estimated ranges are provided in Suppl. Table S2. Stem lineages with solid lines received nodal support values (posterior probabilities, PP) ≥ 0.9, dotted stem lineages received PP < 0.9.

Zealand and one from mainland Australia to New Caledonia, most of which were inferred to have occurred during the Pleistocene (Fig. 5). In one case, i.e. the MRCA of the "venosa" clade, the range evolutionary scenario involving a distribution in New Zealand remained ambiguous due to the unresolved ancestral range of the MRCA of *Thelymitra*. Multiple range expansions and range shifts to Tasmania were inferred, mostly from mainland Australia, and predominantly of relatively recent origin, in the Pleistocene or later (Fig. 5). Exact range estimates for each node are provided in Suppl. Table S2; a more detailed description of the biogeographic history of all major clades with remarks on their sampling is given in Supplement A.

## 4. Discussion

### 4.1. Phylogenetic relationships

This study clarified intergeneric relationships within Thelymitrinae, providing strong support for *Epiblema* as sister group to *Thelymitra* and *Calochilus*. Our phylogenetic analysis based on plastid and nuclear regions supported the monophyly of *Thelymitra* and *Calochilus,* as well as their sister group relationship to each other. Previous phylogenetic studies remained inconclusive regarding relationships within Thelymitrinae either due to limited sampling (Kores et al., 2001; Freudenstein et al., 2004) or due to conflicting signal between nuclear and plastid markers (Clements et al., 2002; Weston et al., 2014). Early studies based on ITS showed a sister group relationship between

*Calochilus* and a clade comprising *Thelymitra* and *Epiblema*, with the latter being embedded within *Thelymitra* (Clements et al., 2002). A molecular study based on plastid and nuclear datasets retrieved incongruent relationships within Thelymitrinae (Weston et al., 2014). While the phylogenetic reconstruction based on ITS in Weston et al. (2014) was congruent with the ITS study by Clements et al. (2002), the inferred plastid phylogeny showed *Epiblema* as the first diverging lineage and *Thelymitra* and *Calochilus* as sister groups with moderate to high statistical support. However, both studies used the same ITS sequence for *Epiblema* (GenBank accession AF348029) (Clements et al., 2002; Weston et al., 2014).

For a critical assessment of the discrepancies between the studies, we included the previously published ITS sequence in our dataset along with an additional *Epiblema* ITS sequence we generated (S1079). This revealed that the published ITS sequence had close affinities with *T. cucullata*, whereas the newly generated two *Epiblema* sequences grouped together and were placed in sister group position to *Thelymitra* and *Calochilus* (results not shown). We therefore conclude that the first published ITS sequence on *Epiblema* was erroneous. The topologies retrieved with the correct ITS sequences for *Epiblema* and the plastid markers are congruent. Thus, our study clarified the phylogenetic position of *Epiblema* as the first diverging lineage within Thelymitrinae, sister to the monophyletic genera *Thelymitra* and *Calochilus*.

This study also provided the first insights into intrageneric relationships in *Thelymitra*. It included representatives from all major morphological groups recognized within *Thelymitra* and represented c. 60% of the species diversity found within the genus. Eight major clades were identified as well as several smaller clades. Five major clades corresponded to species groups previously characterised by morphological characters, i.e., the canaliculata, fuscolutea, variegata, and venosa complexes (Jeanes, 2001, 2006, 2009, 2012), and species group 5 (Jones, 2006). The other main clades largely or partly corresponded to previously recognized species groups, i.e., the aristata, nuda, and pauciflora complexes (Jeanes, 2004, 2011, 2013), and species groups 10 and 12 (Jones, 2006), hence were found to be paraphyletic.

Several incongruences were detected between the nuclear and plastid phylogenetic reconstructions, providing evidence for past hybridization events between species belonging to different major lineages. Within the genus, many natural hybrids are known for which parental species occur in sympatry and which have morphological similarities to both parental species, e.g., *Thelymitra* × *chasmogasma*, *T.* × *irregularis*, and *T.* × *macmillanii* (Jones, 2006). Cytogenetic studies in *Thelymitra* established a chromosome base number of 2n = 28 for the genus and documented a wide range of chromosome numbers and different ploidies (e.g., 2n = 26, 40, 45, 54, 57, 62, 70, 84, 93), pointing to past hybridization events involving allopolyploidy and/or dysploidy (Dawson et al., 2007).

The incongruences detected in our molecular study corroborate previous hypotheses on the origin of species within *Thelymitra* through hybridisation. For instance, Dawson et al. (2007) hypothesised that *T. carnea* and *T. rubra*, each with a chromosome number of 2n = 62, are allopolyploid hybrids between *T. flexuosa* and *T. pauciflora,* which possess chromosome numbers of 2n = 36 and 2n = 26, respectively. The incongruences between the inferred nuclear and plastid phylogenies detected in our study provide further support for this hypothesis. In the nuclear trees, *T. rubra* and *T. carnea* grouped with *T. flexuosa,* which is congruent with their close morphological affinity to *T. flexuosa*, while their position in the plastid trees provided evidence for past hybridization with members of the “pauciflora/nuda” clade.

In several cases, supported incongruences were found in the positioning of clades. This points to hybridisation events in the deeper evolutionary history of the genus. The most striking incongruence was found in the position of the “variegata” clade. In the nuclear trees, the “variegata” clade formed a sister group to *T. crinita*, and this clade was found in sister group position to the “pauciflora/nuda” clade. In the plastid trees, the “variegata” clade was resolved as sister to the “antennifera” clade. These results point toward an ancient hybridisation event between the “antennifera” clade and the “pauciflora/nuda” clade or *T. crinita* with subsequent diversification of the resultant lineage, the “variegata” clade. The species of both clades show morphological similarities, and in the past, several of them were grouped together in the formerly recognized genus *Macdonaldia* Gunn ex Lindl. Our results showed that *Macdonaldia* does not constitute a monophyletic group and therefore its recently proposed resurrection (Szlachetko, 1995) is not supported by our findings.

Another incongruence was found in the position of a clade formed by *T. cornicina* and *T. campanulata.* This clade was embedded within the “pauciflora/nuda” clade in the nuclear trees and sister to the “ixioides” clade in the plastid trees. These results indicate past hybridisation between species from the “ixioides” and the “pauciflora/nuda” clades.

The “canaliculata” clade also exhibited an incongruent phylogenetic position between the nuclear and plastid trees. In the nuclear trees it was found in sister group position to the “aristata” clade, whereas in the plastid trees it was found in sister group position to a clade formed by *T. crinita*, *T. mucida,* and the “pauciflora/nuda” clade. This indicates a past hybridisation event between species of these two lineages. In these last two cases, chromosome counts for the species are not available. Further cytogenetic studies in *Thelymitra* are required to enhance our understanding of the importance of hybridisation, allopolyploidy, and dysploidy in the evolution of *Thelymitra.*

### 4.2. Diversification during the Neogene and Quaternary

This study provided the first divergence time estimation for *Thelymitra*. The origin of *Thelymitra* and the onset of the crown diversification of the genus were placed in the late Miocene, around 10.8 Ma and 6.1 Ma, respectively. In Australia, a global cooling trend, a lowered sea level and a major ice sheet on Antarctica led to cooler and drier conditions during the late Miocene than during the early Miocene (Martin, 2006). This resulted in marked changes in the vegetation cover of the continent, with a decline of closed rainforest and increased expansion of wet sclerophyll forests dominated by *Eucalyptus*, other Myrtaceae, and Casuarinaceae (Kershaw, 1994; Martin, 1994). An abundance of charcoal sediments in late Miocene deposits provide evidence for the presence of a well-marked dry season in the southern part of Australia allowing for more frequent burning to the extent that by this time burning had become part of the environment (Martin, 1987, 2006).

The changes in climate and environmental conditions of the late Miocene can be regarded as beneficial for the geographic expansion and diversification of *Thelymitra* due to the increase in suitable habitats, i.e. open vegetation communities, and the more regular occurrence of natural fires. Fire reduces the abundance of competing above ground vegetation, releases nutrients into the soil, and stimulates flowering of many terrestrial orchids of the Australian flora, including *Thelymitra* species (Lamont and Downes, 2011; Calder et al., 1989; Norton and De Lange, 2003; Coates et al., 2006). The geophytic life form of *Thelymitra* with its underground dormancy during the drier and hotter summer months (a time of the year which is also more prone to natural fires) may have been an advantageous feature of *Thelymitra* species, enabling them to cope with and benefit from the climatic and environmental changes from the late Miocene onwards.

Our divergence time estimates showed that the majority of main clades in *Thelymitra* arose during the late Pliocene and early Pleistocene. While the early Pliocene was characterised by warmer and more stable climatic conditions in Australia, the climate of the late Pleistocene was cooler and more variable (Gallagher et al., 2003). Increased aridification of the continent resulted in a marked acceleration of the transition from rainforest vegetation to open sclerophyll forests (Hill et al., 1999); open grasslands emerged and expanded for the first time, with Poaceae and Asteraceae increasing in abundance in the fossil record (Martin, 1998, 2006). By the Pleistocene, the vegetation of

Australia had mostly changed to open woodlands and grasslands (Martin, 1987; Kershaw, 1994). These broad scale vegetation changes during the late Pliocene and early Pleistocene led to a significant expansion of suitable environments for *Thelymitra* and thus provided increased opportunities for fine scale ecological differentiation within the genus.

The divergence time estimation revealed that the majority of extant *Thelymitra* species originated in the Pleistocene and thus are of remarkably young age. The Pleistocene was characterized by frequent and strong climatic oscillations, which can be seen as an important factor driving speciation in *Thelymitra.* During the more humid conditions of the glacial periods, denser vegetation communities expanded, which reduced the extent of suitable habitats for *Thelymitra*. During the drier conditions of the interglacials, suitable habitat for *Thelymitra* such as grasslands, open heath, and mallee eucalypt shrubland communities increased (Martin 2006). This facilitated repeated cycles of isolation and secondary contact of previously isolated *Thelymitra* populations, promoting speciation. Further, several factors in the biology of *Thelymitra* can be seen as supportive for speciation under the conditions of the Pleistocene: (1) a generalized pollination syndrome and the apparent low pre-and postzygotic barriers, which foster the creation of hybrids when previously separated populations expand and form sympatric stands, (2) the frequency of alloploidy and aneuploidy, which create highly varied genomic attributes in *Thelymitra* that can result in a broader ecological amplitude of polyploids relative to the diploid parents (e.g., Arnold and Martin, 2010; Spoelhof et al., 2017), and (3) the frequent occurrence of self-compatibility and increased heterozygosity, which are regarded to support colonization of new ecological niches and survival when population sizes decrease (Soltis and Soltis, 2000).

### 4.3. Spatio-temporal evolution

The ancestral range analysis provided new insights into the evolution of today's distributional patterns in *Thelymitra*. While the geographic origin of the genus could not be reconstructed with confidence, the ancestral range of the first diverging lineage, the "venosa" clade, was reconstructed as eastern (eastern Australia, Tasmania, and New Zealand) whereas the ancestral area for the remainder of the genus was reconstructed as southwest Australian.

This first divergence in *Thelymitra* was dated to the late Miocene (c. 6.1 Ma), when the biogeographic barrier between southwest and southeast Australia, the Nullarbor Plain, was already established, and New Zealand had long been separated from the Australian landmass. The Nullarbor Plain is an area of c. 1000 km semi-arid and almost treeless desert on limestone bedrock, which was established in its current form since the last marine incursion c. 14 Ma, in the mid Miocene. The barrier has been identified to have driven lineage divergence through vicariance in several Australian plant groups, such as *Allocasuarina,* Fabaceae, and Poales (Crisp and Cook, 2007).

However, the inferred age of the first lineage divergence in *Thelymitra* post-dates the emergence of the Nullarbor Plain. This suggests that this distributional pattern is the result of a long-distance dispersal event between southwest Australia and the east. Our biogeographic analysis inferred several subsequent range shifts and expansions from southwest Australia across the Nullarbor Plain to the east of Australia, e.g., in the "canaliculata"/"aristata" clades (from c. 3.1 Ma onwards), the "pauciflora/nuda" clade (from c. 1.9 Ma), and the "variegata" clade (from c. 0.4 Ma onwards), which can be best explained by long-distance dispersal.

Our study showed that early lineage diversification in *Thelymitra* predominantly took place in southwest Australia, in particular during the Pliocene. Further eastward dispersals and subsequent diversification events in the newly occupied areas were predominantly of more recent origin, from the early Pleistocene onwards. The prevalent direction of dispersal in *Thelymitra* was reconstructed to be from the west to the east. Previous biogeographic studies in Australasian plant groups also detected a similar predominant dispersal from the west to the east, which is explained by the prevailing westerly winds in the Southern Hemisphere (Sanmartín et al., 2007). This is also highly likely for the wind-dispersed, dust like seeds of *Thelymitra.* Hence our study provided another example for the strong influence of the westerly winds on Australasian plant distribution patterns.

Our results present strong evidence that the contemporary *Thelymitra* flora of New Zealand was assembled through several independent long-distance dispersals from Australia in recent geological times. At least seven long-distance dispersals to New Zealand were reconstructed from the Pleistocene onwards. This indicates a relatively young age of the *Thelymitra* flora of New Zealand. The biogeographic analysis also indicated that the *Thelymitra* flora of Tasmania was relatively young. We reconstructed at least 13 colonization events of Tasmania, five of which occurred from the early Pleistocene onwards and six from the late Pleistocene onwards.

The relatively young age of the *Thelymitra* floras of New Zealand and Tasmania seems surprising given the evident long distance dispersal capabilities of *Thelymitra* and the presence of the West Wind Drift since the late Eocene. This relatively young age of the *Thelymitra* floras of New Zealand and Tasmania is therefore more likely due to earlier extinctions. During the Plio- and Pleistocene, Tasmania and New Zealand were more severely affected by the cold conditions during glacial periods, which included glaciations in both regions (Martin, 2006; Newnham et al., 1999). On the Australian mainland, in contrast, glaciations were only present in the southeastern highlands (Martin, 2006), thus providing greater opportunities for *Thelymitra* to persist in climatic refugia during the glacial periods of the Plio- and Pleistocene. Given the dispersal capabilities of *Thelymitra* and the high number of colonisations of Tasmania and New Zealand in relatively recent times, we consider early establishment of *Thelymitra* lineages followed by extinction plausible. In the case of New Caledonia, our data support a long-distance dispersal event from eastern Australia, which gave rise to the New Caledonian species *T. sarasiniana*. The inferred age for this divergence event was dated to the late Pleistocene (c. 0.5 Ma), thus the occurrence of *T. sarasiniana* in New Caledonia is also of relatively recent origin.

The *Thelymitra* species occurring in New Guinea (*T. papuana*), Indonesia (*T. javanica*), and the Philippines (*T. forbesii*) were not available for this study, and hence their phylogenetic placement remained uncertain. Based on their close morphological affinities with species from the pauciflora and nuda complexes, we regard it as likely that they are part of the "pauciflora/nuda" clade. As the latter was estimated to have arisen in the late Pliocene, a relative recent origin is also assumed for the occurrences of *Thelymitra* in New Guinea, Indonesia, and the Philippines.

## 5. Conclusions

This study clarified intergeneric relationships in Thelymitrinae and provided first insights into phylogenetic relationships of *Thelymitra* and its spatio-temporal evolution. Divergence dating and ancestral range estimation revealed that *Thelymitra* originated in the late Miocene. Most major lineages originated during the Pliocene in southwest Australia, from where subsequent eastwards long distance dispersals followed. Eastern Australia was reached in the early Pleistocene, and Tasmania, New Zealand, and New Caledonia were colonized from the late Pleistocene onwards. Incongruences between inferred nuclear and plastid phylogenies indicate that hybridization played an important role in the evolutionary history and diversification of *Thelymitra*, in particular under the changing environmental and climatic conditions of the Pleistocene.

While this study provided first hypotheses around evolutionary relationships and the historical biogeography of *Thelymitra*, its complex evolutionary history warrants further study. Recent technological advances in the field of phylogenomics, such as exome capture and

genotyping-by-sequencing, now allow for the generation of large genomic datasets from both nuclear and plastid genomes. In combination with cytogenetic and morphological studies in *Thelymitra*, these will further enhance our understanding of the complex evolutionary relationships in the genus, including the delimitation of closely related species in this young group of orchids. In the face of diverse anthropogenic pressures on habitats of *Thelymitra*, these studies are urgently required to provide a scientific framework for the re-evaluation of the conservation status of rare and endangered *Thelymitra* species and to inform their effective conservation management.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2018.05.031.

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