Delpinoa **56-57**: 105-113. 2014-2015

DNA barcoding analysis of *Eucalyptus camaldulensis* Dehnh. (Myrtaceae)

A. DE LUCA[1], G. SIBILIO[2], E. DEL GUACCHIO[2]

[1]Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Via F. Delpino 1, 80139 Naples, Italy.
[2]Botanical Garden of Naples, University of Naples Federico II, Via Foria 223, 80139 Naples, Italy.
adriana.deluca@unina.it, gsibilio@unina.it, edelgua@email.it

Riassunto. La nomenclatura di *Eucalyptus camaldulensis* Dehnh. (Myrtaceae) è stata molto discussa poichè studi moderni suggerivano che Dehnhardt, l'autore della specie, avesse utilizzato questo nome per una pianta diversa da quella attualmente indicata con questo nome. Un recente lavoro, condotto con un approccio multidisciplinare basato sull'esame di illustrazioni e campioni di erbario nonché sulla consultazione di documenti in archivi storici, ha mostrato che la descrizione originale di Dehnhardt è applicabile al taxon correntemente indicato come *E. camaldulensis* subsp. *camaldulensis*. Per supportare questa conclusione, è stata condotta un'analisi del DNA barcoding su esemplari storici, coltivati o selvatici di *E. camaldulensis*. I risultati hanno confermato che non esiste una differenza tassonomica sostanziale tra la pianta su cui Dehnhardt ha descritto *E. camaldulensis* e le piante in seguito indicate con questo nome.

Abstract. The nomenclature of *Eucalyptus camaldulensis* Dehnh. (Myrtaceae) has been much-discussed since modern studies suggested that Dehnhardt, the author of the species, employed this name for a plant different from that indicated today with this named. A recent work carried out by a multidisciplinary approach based on examination of illustrations and herbaria specimens as well as on consultation of documents in historical archives, showed that Dehnhardt's original description applies to the taxon currently indicated as *E. camaldulensis* subsp. *camaldulensis*. In order to support this previous conclusion, a DNA barcoding analysis was carried on both historical specimens and cultivated and wild material of *E. camaldulensis*. Results confirmed that there is no substantial taxonomical difference between the plant on which Dehnhardt established *E. camaldulensis* and the plants now indicated by this name.

Key Words: DNA barcoding analysis, *Eucalyptus camaldulensis*, Typification

INTRODUCTION

Eucalyptus camaldulensis Dehnh. (Myrtaceae) is probably the most common *Eucalyptus* both in its native range (Australia) and worldwide. The species was described by Friedrich Dehnhardt, a distinguished German botanist who worked in the 19th century in various historical gardens of the city of Naples, including the Botanical Garden of Naples and the no longer existing *Hortus Camaldulensis* (Garden of Camaldoli), so called from the name of his owner Francesco Ricciardi, Count of Camaldoli, a town hill in front of Naples (BARONE LUMAGA & MENALE 2000; MENALE & BARONE LUMAGA 2000). From the name of this garden, the specific epithet *camaldulensis* derives. In all these historical gardens, plants of *Eucalyptus camaldulensis* were cultivated, the great part of which have now disappeared (DEL GUACCHIO *et al.* 2019).

Current opinion suggested that Dehnhardt applied this name to a plant different from that to which the name has commonly been applied; hence, *E. camaldulensis* was conserved with a conserved type (BARRIE 2011). Despite this, in a previous work, by a multidisciplinary approach based on examination of illustrations and herbaria specimens as well as on consultation of documents in historical archives, DEL GUACCHIO *et al.* (2019) showed that Dehnhardt's original description clearly applied to the modern delimitation of

E. camaldulensis subsp. *camaldulensis* and that conservation of the name was not necessary (DEL GUACCHIO *et al.* 2019).

In the present work, in order to give support to conclusions included in the previous nomenclatural investigations (DEL GUACCHIO *et al.* 2019), we have subjected to molecular investigations various samples of *E. camaldulensis* examined in the previous work and of two related species considered by us to be of interest in this regard.

MATERIALS AND METHODS

A DNA barcoding analysis was carried on to compare the DNA from a specimen from historical collection, from two old plants cultivated in Naples and identified as *E. camaldulensis* and from eight specimens prepared from individuals growing in the wild in the native range in Australia, including a duplicate of the neotype (EU_CAM_A_5156, Table 1).

Total DNA was extracted following the CTAB protocol (DOYLE & DOYLE 1987), using approx. 50 mg of dehydrated leaf tissue, one chloroform-isoamyl alcohol (24:1) purification step and an over-night precipitation at -80 °C in isopropyl alcohol. Then, the genomic DNA extracted was again purified using the Monarch Nucleic Acid Purification Kit (New England Biolabs) and re-suspended in 15 µL of molecular biology grade water. PCR reactions were prepared for the following DNA barcoding markers (HOLLINGSWORTH *et al.* 2011): Internal Transcribed Spacer 2 (ITS2), from nuclear DNA (nrDNA), and the variable part of *mat*K gene and *psb*A-*trn*H$^{(GUG)}$ intergenic spacer, from chloroplast DNA (cpDNA). Molecular markers were amplified by using primers reported in Table 2. The volume of each amplification reaction was 25 µL, using 5 ng of template, 12.5 µL of Platinum SuperFi PCR Master Mix (Thermo Fisher Scientific Inc.) and 0.125 µL of 25 µM primers. The cycling parameters were: initial denaturation at 98° C for 30 s, followed by 30-35 cycles of denaturation at 98 °C for 7 s, annealing for 10 s at a temperature depending on the Tm of the primers used, and extension at 72 °C for 30 s. A final extension at 72 °C for 5 min was included. Amplified products were purified by 20% polyethylene glycol (PEG) 8000 precipitation (AppliChem GmbH), and purified templates were sequenced by using a fluorescent dye (Bright Dye Terminator Cycle Sequencing Kit, ICloning) according to the manufacturer's instructions. The reactions were purified using BigDye XTerminator Purification Kit (Applied Biosystems, Thermo Fisher Scientific) and read using an automated sequencer (3130 Genetic Analyzer, Life Technologies, Thermo Fisher Scientific). The sequences raw data were analysed with the AB DNA Sequencing Analysis ver. 5.2 software (Applied Biosystems, Thermo Fisher Scientific). Electropherograms were edited using Chromas Lite ver. 2.1.1. software (Technelysium), assembled and aligned with Chromas Pro ver. 2.1.5 (Technelysium) and BioEdit ver. 7.2.5 software (HALL 1999). A standard IUPAC ambiguity code was assigned if nucleotide peaks were overlapping and the lower peak was at least one third in height as the highest one (Y for C-T; W for A-T; R for A-G; S for C-G; M for A-C; K for G-T). The identification of sequence barcodes from the *Eucalyptus* samples was conducted using the Basic Local Alignment Search Tool (BLAST; ALTSCHUL *et al.* 1997). To optimize correct identifications, the closest match for each molecular marker was defined as the target with the highest percentage identity using an arbitrary cut-off of 98% and an E-value < 1e-4 or greater in terms of overlap with the query sequence.

After initial experiments showed similarity between the sequences of *E. camaldulensis* and those available in the literature, five specimens of *E. grandis* W.Hill (nomenclature according to BEAN 2002) and three of *E. tereticornis* Sm. were sequenced and included in the analyses (Table 1).

RESULTS AND DISCUSSIONS

Sequences for the investigated samples are quite similar to one another. In particular, nDNA ITS2 showed eight different positions (positions 16, 28, 32, 34, 38, 82, 84, and 186 of the alignment), mainly characterised by paralogies across several samples (all alignments are available upon request to the author A. De Luca); with the exclusion of pos. 16, in which sample EUCAM_A_5156

shows either A or C (i.e., M) and all other samples either show C or T or the corresponding paralogue Y, all the other paralogous positions are additive across the samples. Samples from Naples are paralogous at pos. 32 and 186 (all three) as well as at pos. 34 (only sample TV_NAP).

The cpDNA psbA-trnH intergenic spacer, which is the most variable sequence employed, in addition to sixteen-point mutations (positions 104, 125, 137, 143, 155, 191, 193, 202, 216, 241, 248, 250, 285, 327, 358, 359, shows four length mutations. Two of them are represented by a poly-T stretch (starting at pos. 173 of the alignment) and by a poly-A stretch (starting at pos. 380). The other two length mutation are a 27 bp indel (starting at pos. 252 of the alignment) and a 7 bp indel (starting at pos. 360 of the alignment).

In cpDNA matK, seven positions differ (namely, positions 73, 113, 397, 796, 810, 980, and 1016 of the alignment). Positions 113 and 980 are uninformative (variation only occurs for EU_CAM_A_RJMM17 and for EU_GR_A_22948, respectively), whereas all the others show informative differences. For matK, only sample EU_TERET_A_14200 was sequenced, given the lower rate of variation of this sequence as compared to the others.

A BLAST search of the obtained *E. camaldulensis* sequences showed that various ITS2 sequences of our species, including those of the Neapolitan specimens, are equally related to *E. camaldulensis* as to *E. tereticornis*, with the exception of EU_CAM_A_RJMM17 (identified as *E. camaldulensis* subsp. *acuta*), which is 100% identical to a *E. camaldulensis* target sequence. Cross-species identity is shown also for some of our samples of *E. grandis* and *E. tereticornis*.

A separate BLAST search on the two chloroplast DNA sequences of all the investigates specimens of *E. camaldulensis* showed that several of them are related to *E. grandis* chloroplast DNA more than to that of *E. camaldulensis* for *mat*K only one specimen of *E. tereticornis* was sequenced, as the latter sequence is known to be much more conserved than *trn*H$^{(GUG)}$-*psb*A). Two neighbour-joining analyses run separately on two distances matrices of the ITS2 (Fig. 1) and chloroplast DNA sequences (Fig. 1) showed that in neither case could the sequences of *E. camaldulensis* or those of *E. grandis* be recovered as a single "monophyletic" cluster (to obtain a merged chloroplast DNA alignment for this analysis, samples EU_TERET_A_4670 and EU_TERET_A_12316, which were sequenced for the cpDNA psbA-trnH intergenic spacer, were considered as missing data for matK).

The specimens in which we are most interested, i.e., the isoneotype EU_CAM_A_5156 (which is by definition subsp. *camaldulensis*, the specimen collected by Gussone in *Hortus camaldulensis* (EUCAM DEN) and the two Dehnhardt related specimens from Naples (TV NAP and VC NAP), all identified as the same subspecies, do not have identical ITS2 sequences and cannot be recovered in a single cluster using our nuclear DNA sequences, either rooting the tree with *E. tereticornis* or with *E. grandis* (in fact, the dendrogram in Fig. 1 is midpoint rooted). However, given the high similarity of the sequences and their paralogies, we avoid further comments.

On the contrary, the four just mentioned specimens, i.e., EU_CAM_A_5156, EUCAM DEN, TV NAP and VC NAP, all identified as the same subspecies, have identical chloroplast DNA sequences and are recovered in a single cluster, together with EU_CAM_30771 (Fig. 2). However, the immediately most inclusive cluster contains also *E. grandis* 18518 and 32319 and then also Eucam 27745 and *E. grandis* 19050.

The results shown here are a further proof that *E. camaldulensis*, as numerous other species of the genus (GRIFFIN *et al.* 1988; MCKINNON *et al.* 1999; POTTS *et al.* 2011), frequently hybridise and introgress with their congeners. In particular, in addition to intergradation between subspecies (in particular, *E camaldulensis* subsp. *acuminata* and subsp. *obtusa*), populations of our species (in particular, subsp. *arida*) have been found to intergrade with *E. rudis* Endl. (BUTCHER *et al*. 2009). One of the possible explanations of the widespread hybridisation in the genus, at least between species with very different ecological requisites, is related to responses to rapidly changing abiotic conditions

Table 1 - List of *Eucalyptus* accessions sequenced, voucher information and origin.

Code	Taxon	Origin (Collection date)	Voucher (Herbarium)
EUCAM_DEN	*Eucalyptus camaldulensis* Dehnh.	*Hortus Camaldulensis* of Naples, Italy (1849)	Gussone s.n. (NAP, coll. Gussone "Generale")
TV_NAP	*E. camaldulensis*	Villa Comunale, Naples, Italy (14/06/2016)	G. Sibilio s.n. (NAP)
VC_NAP	*E. camaldulensis*	Villa Comunale, Naples, Italy (14/06/2016)	G. Sibilio s.n. (NAP)
EU_CAM_A_5156	*E. camaldulensis*	Bridge on Strathalbyn to Goolwa Road, Australia (14/02/2008)	Nicolle 5156 (BRI)
EU_CAM_A_12348	*E. camaldulensis*	Aberdeen town near Golf course, Australia (10/10/1995)	Brooker 12348 (BRI)
EU_CAM_A_27745	*E. camaldulensis*	Talbragar River, Australia (27/03/2008)	Bean 27745 (BRI)
EU_CAM_A_30761	*E. camaldulensis*	2.2 km from Warwick-Inglewood road, Australia (28/02/2011)	Bean 30761 (BRI)
EU_CAM_A_RJMM17	*E. camaldulensis* subsp. *acuta* Brooker & M.W.McDonald	Murra Murra, Australia (27/05/2007)	Eddie RJMM17 (BRI)
EU_CAM_A_5094	*E. camaldulensis* subsp. *acuta*	Powlathanga Station, Australia (28/10/2008)	Booth 5094 (BRI)
EU_CAM_A_1220	*E. camaldulensis* subsp. *arida* Brooker & M.W.McDonald	Tickalara Homestead, Australia (14/04/2008)	Turpin 1220 (BRI)
EU_CAM_A_1127	*E. camaldulensis* subsp. *obtusa* (Blakely) Brooker & M.W.McDonald	Bladensberg National Park, Australia (13/09/2009)	Gandini 1127 (BRI)
EU_GR_A_22948	*E. grandis* W.Hill	Daintree National Park, Australia (24/05/1998)	Forster 22948 (BRI)
EU_GR_A_21166	*E. grandis*	Paluma Dam, Australia (02/10/2002)	Cumming 21166 (BRI)
EU_GR_A_32319	*E. grandis*	NW of Boreen Point, Australia (05/06/2015)	Bean 32319 (BRI)
EU_GR_A_19050	*E. grandis*	Logan River, Australia (09/06/2002)	Bean 19050 (BRI)
EU_GR_A_18518	*E. grandis*	Ormeau, Australia (10/02/2002)	Bean 18518 (BRI)
EU_TERET_A_4670	*E. tereticornis* Sm.	74 km N of Lynd Junction, Australia (03/07/1994)	Hill 4670 (BRI)
EU_TERET_A_14200	*E. tereticornis*	45 km SSE of Springsure, Australia (14/10/1998)	Bean 14200 (BRI)
EU_TERET_A_12316	*E. tereticornis*	Warrigal Range Rd, Australia (12/09/1995)	Brooker 12316 (BRI)

Table 2 - Primers used for DNA barcoding analyses.

Locus	Primer name	Sequence (5'-3')	Ta	Reference
cpDNA				
***mat*K**	EuMatK-F	TAT GCA CTT KCT CAT GAT CA	50	THORNHILL *et al.* 2015 (forward modified for an addition of K)
	Eu_MatK-R	TTT ACG AGC CAA AGT TTT AA		
***trn*H$^{(GUG)}$-*psb*A**	psbA3'f	GTT ATG CAT GAA CGT AAT GCT C	55	SANG *et al.* 1997
	trnHf	CGC GCA TGG TGG ATT CAC AAT CC		TATE & SIMPSON 2003
nrDNA				
ITS	Forward: 18S-3' (JK14)	GGA GAA GTC GTA ACA AGG TTT CCG	55	ACETO *et al.* 1999
	Reverse: 26S-5′ (SN3)	TTC GCT CGC CGT TAC TAA GGG		DE CASTRO *et al.* 2013
	EU_ITS1_for*	AGC AGA ATG ACC AGA GAA CC		This study
	EU_ITS2_rev*	CGC GCG ACA TTG ATC ATT G		

* Used also for nested PCR.

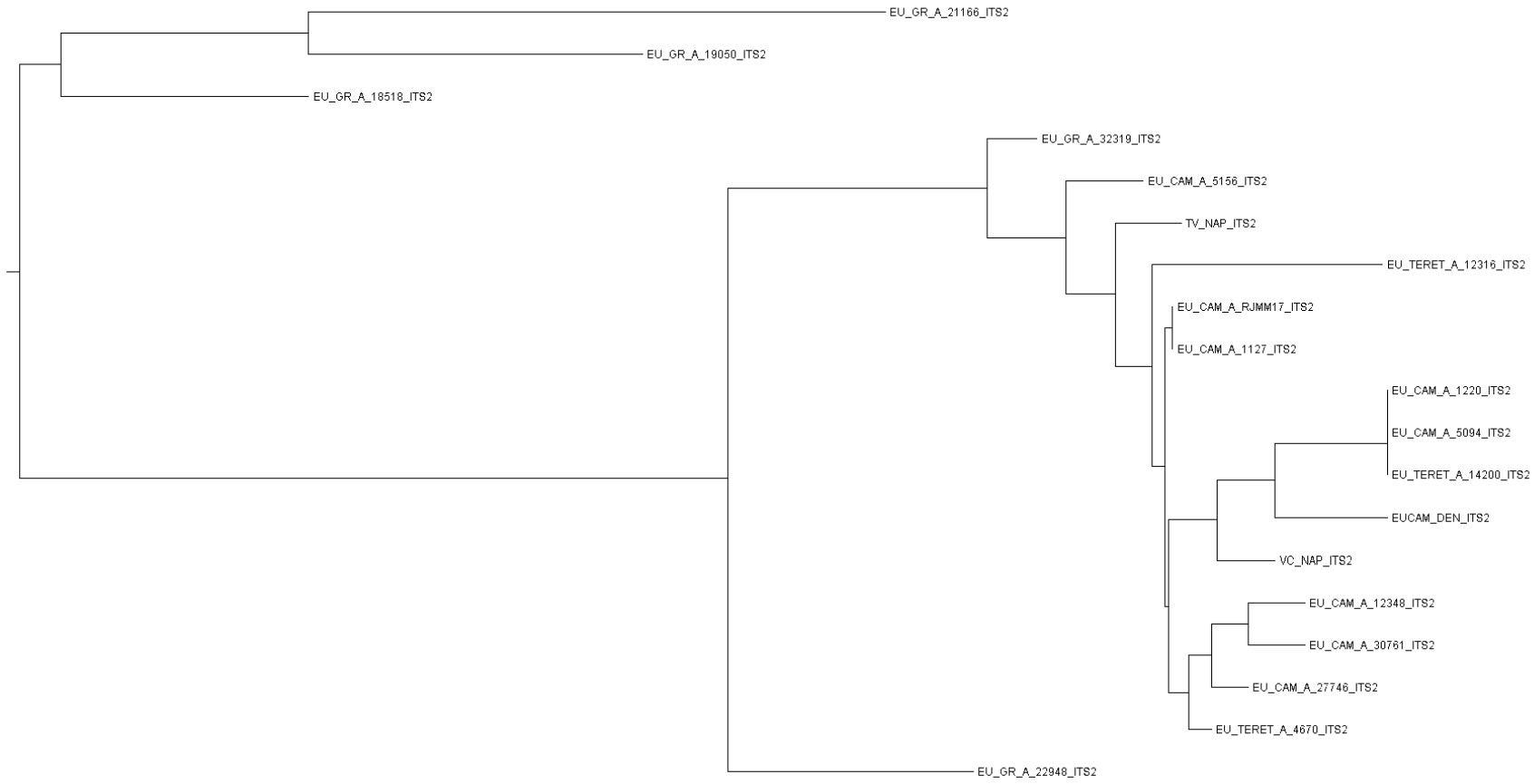

Fig. 1 - Neighbor-Joining dendrogram of the ITS2 sequences. In the lack of monophyletic units, the tree was midpoint-rooted.

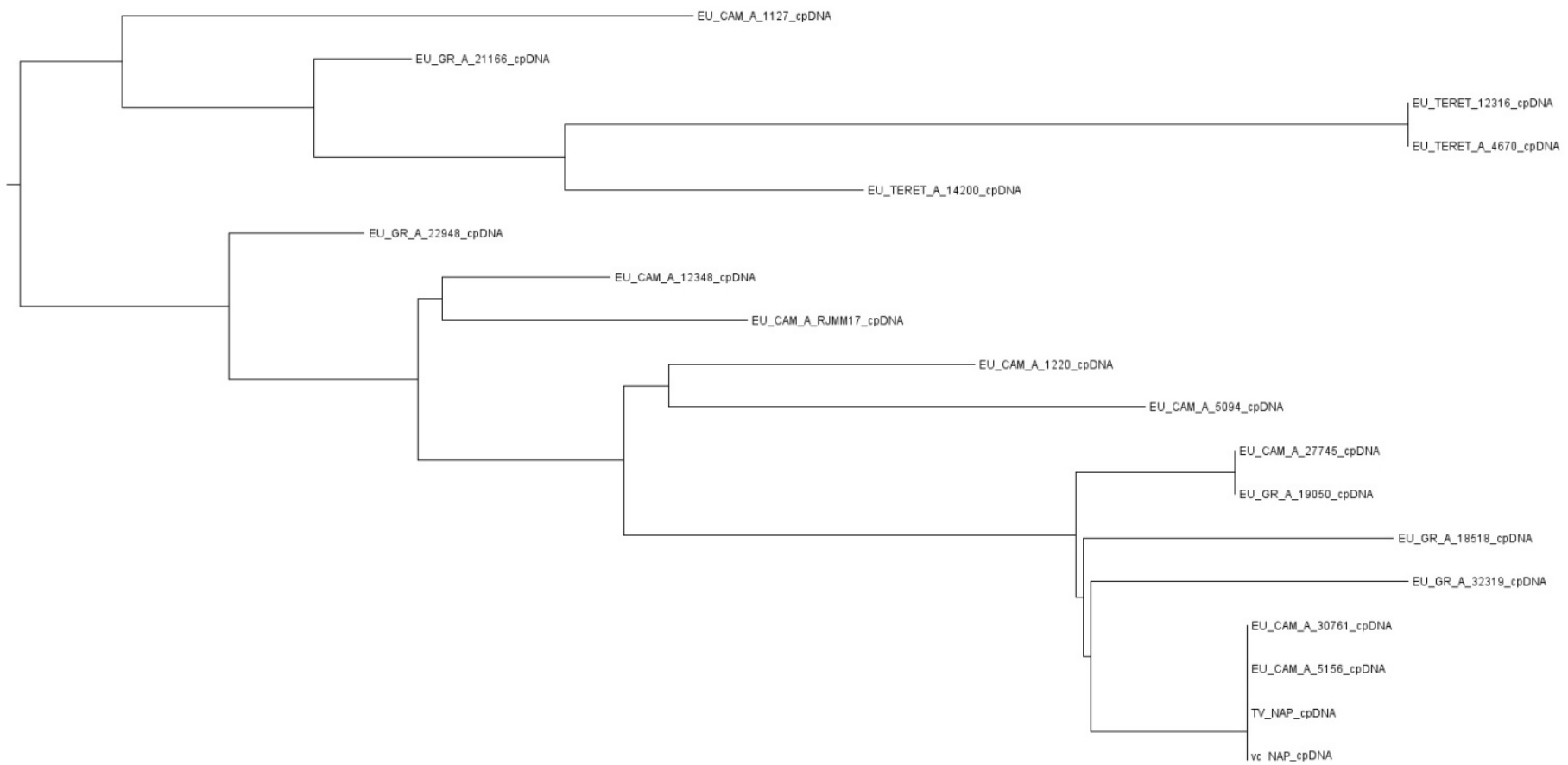

Fig. 2 - Neighbor-Joining dendrogram of the chloroplast DNA sequences

(NEVILL *et al.* 2014). In the case of *E. camaldulensis*, however, reticulation is one of the characteristic of the species. In fact, as JONES *et al.* (2016) clearly showed, in a large study dealing with over 500 specimens for 185 terminal taxa, using 3109 DArT markers, *E. camaldulensis* does not fall in a single clade within *Eucalyptus*, and its different subspecies cluster with various other different taxa.

Obviously, given the small sample investigated, we refrain from any speculation on the matter, not being able to understand whether the common chloroplast sequences between our species derive from direct hybridisation/introgression between the three taxa or they share chloroplast sequences for more complex historical reasons. However, all three species can be easily artificially hybridised (e.g., SUMATHI & RAMASAMI 2017) and they appear in a previous study by STEANE *et al.* (2011) as not very distant from one another in terms of phylogeny.

Indeed, even if the molecular results of the present investigation might be construed as a failure of DNA barcoding in species identification in *Eucalyptus*, we would like to point out that the three Neapolitan specimens and the isoneotype have identical chloroplast DNA sequences, shared only by another specimen (EU_CAM_30771) in our inclusive sample. Moreover, the investigation of barcoding sequences reported here returned a blurred picture of hybridization and introgression which, by itself, would behave as a most evident clue encouraging further studies by using other markers. These studies have in fact been carried out (see JONES *et al.* 2016 and references therein), confirming that the success of barcoding, at least in vascular plants, heavily depends upon the evolutionary history of the investigated group (WYLER & NACIRI 2016).

LITERATURE CITED

ACETO S., CAPUTO P., COZZOLINO S., GAUDIO L., MORETTI A. 1999. Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. Mol. Phylogenet. Evol. 13: 67-76. http://doi.org/10.1006/mpev.1999.0628

ALTSCHUL S.F., MADDEN T.L., SCHAÈFFER A.A., ZHANG J., ZHANG Z., MILLER W., LIPMAN J.D. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

BARONE LUMAGA M.R., MENALE B. 2000. L'*Hortus Camaldulensis*. Delpinoa 42: 43-45.

BARRIE F.R. 2011. Report of the General Committee: 11. Taxon 60: 1211-1214. http://www.jstor.org/stable/41317343

BEAN A.R. 2002. A note on the authorship of *Eucalyptus grandis*. Austr. Syst. Bot. Soc. Newsletter 112: 2.

BUTCHER P.A., MCDONALD M.W., BELL J.C. 2009. Congruence between environmenttal parameters, morphology and genetic structure in Australia's most widely distributed eucalypt, *Eucalyptus camaldulensis*. Tree Genet. Genomes 5: 189-210. doi:10.1007/s11295-008-0169-6

DE CASTRO O., VALLARIELLO R., DEL GUACCHIO E. 2013. Integration of morphology, genetics, historical and ethnobotanical data: a case of an enigmatic *Genista* (Fabaceae) from Ischia Island (southern Italy). Phytotaxa 80: 64-68. http://dx.doi.org/10.11646/phytotaxa.82.2.2

DEL GUACCHIO E., RUSSELL BEAN A. SIBILIO G., DE LUCA A., DE CASTRO O., CAPUTO P. 2019. Wandering among Dehnhardt's gums: The cold case of *Eucalyptus camaldulensis* (Myrtaceae) and other nomenclatural notes on *Eucalyptus*. Taxon 68 (2).

DOYLE J.J., DOYLE J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19: 11-15.

GRIFFIN A.R., BURGES, I.P., WOLF L. 1988. Patterns of natural and manipulated hy-

bridisation in the genus *Eucalyptus* L'Herit. - a Review. Austr. J. Bot. 35: 41-66.

HALL T.A. 1999 [Current version 7.2.6.1 (2017)]. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98.

HOLLINGSWORTH P.M., GRAHAM S.W., LITTLE D.P. 2011. Choosing and using a plant DNA Barcode. PLoS ONE 6 (5): e19254. https://doi.org/10.1371/journal.pone.0019254

JONES R.C., NICOLLE D., STEANE D.A., VAILLANCOURT R.E., POTTS B.M. 2016. High density, genome-wide markers and intra-specific replication yield an unprecedented phylogenetic reconstruction of a globally significant, speciose lineage of *Eucalyptus*. Mol. Phylogenet. Evol. 105: 63-85. https://doi.org/10.1016/j.ympev.2016.08.009.

MCKINNON G.E., STEANE D.A., POTTS B.M., VAILLANCOURT R.E. 1999. Incongruence between chloroplast and species phylogenies in *Eucalyptus* subgenus *Monocalyptus* (Myrtaceae) Am. J. Bot. 86 (7): 1038-1046.

MENALE B., BARONE LUMAGA M.R. 2000. L'Orto Botanico di Napoli e la pianificazione dei Siti Reali: il ruolo di Federico Dehnhardt. Delpinoa 42: 39-41.

NEVILL G.P., DESPRÉS T., BAYLY M.J., BOSSINGER G., ADES P.K. 2014. Shared phylogeographic patterns and widespread chloroplast haplotype sharing in *Eucalyptus* species with different ecological tolerances. Tree Genet. Genomes 10: 1079-1092. https://doi.org/10.1007/s11295-014-0744-y

POTTS B., HAMILTON M., BLACKBURN D. 2011. Genetics of Eucalypts: Traps and opportunities. pp.: 1-27. In: Walker J. (Ed.). Developing a eucalypt resource: learning from Australia and elsewhere. Canterbury (New Zealand): Wood Technology Research Centre (University of Canterbury).

SANG T., CRAWFORD D.J., STUESSY T.F. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Am. J. Bot. 84: 1120-1136.

SUMATHI M., RAMASAMY Y. 2017. Microsatellite allele length variations in inter-specific hybrids of *Eucalyptus*. Acta Bot. Croat. 76 (1): 103-106. https://doi.org/10.1515/botcro-2016-0050

TATE J.A., SIMPSON B.B. 2003. Paraphyly of *Tarasa* (Malvaceae) and diverse origins of the polyploid species. Syst. Bot. 28: 723-737. https://doi.org/10.1043/02-64.1

THORNHILL A.H., HO S.Y.W., KÜLHEIM C., CRISP M.D. 2015. Interpreting the modern distribution of Myrtaceae using a dated molecular phylogeny. Mol. Phylogenet. Evol. 93: 29-43. http://dx.doi.org/10.1016/j.ympev.2015.07.007

WYLER S.C., NACIRI Y. 2016. Evolutionary histories determine DNA barcoding success in vascular plants: seven case studies using intraspecific broad sampling of closely related species. BMC Evolutionary Biology 16: 103. https://doi.org/10.1186/s12862-016-0678-0

Pubblicato nel mese di settembre 2019