10. Technical Questionnaire

TECHNICAL QUESTIONNAIRE	Page {x} of {y}	Reference Number:

	Application date: (not to be filled in by the applicant)

TECHNICAL QUESTIONNAIRE
to be completed in connection with an application for plant breeders' rights

1. Subject of the Technical Questionnaire

1.1 Genus	*Eucalyptus*
1.2 Sub-genus	*Symphyomyrtus*
1.3 Section	Transversaria – Exsertaria - Maidenaria
1.4 Species (please complete)	

2. Applicant

Name	
Address	
Telephone No.	
Fax No.	
E-mail address	
Breeder (if different from applicant)	

3. Proposed denomination and breeder's reference

Proposed denomination (if available)	
Breeder's reference	

TECHNICAL QUESTIONNAIRE	Page {x} of {y}	Reference Number:

#4. Information on the breeding scheme and propagation of the variety

4.1 Breeding scheme

Variety resulting from:

4.1.1 Crossing

(a) controlled cross [ ]
(please state parent varieties)

(b) partially known cross [ ]
(please state known parent variety(ies))

(c) unknown cross [ ]

4.1.2 Mutation [ ]
(please state parent variety)

4.1.3 Discovery and development [ ]
(please state where and when discovered and how developed)

4.1.4 Other [ ]
(please provide details)

4.2 Method of propagating the variety

4.2.1 Vegetative propagation

(a) cuttings [ ]
(b) *in vitro* propagation [ ]
(c) other (state method) [ ]

4.2.2 Seed [ ]

4.2.3 Other [ ]

Authorities may allow certain of this information to be provided in a confidential section of the Technical Questionnaire.

TECHNICAL QUESTIONNAIRE	Page {x} of {y}	Reference Number:

5. Characteristics of the variety to be indicated (the number in brackets refers to the corresponding characteristic in Test Guidelines; please mark the note which best corresponds).

	Characteristics	Example Varieties	Note
5.1 (1)	**Leaf: petiole**		
	absent		1 [ ]
	present		9 [ ]
5.2 (19)	**Primary branch: type of insertion in main stem**		
	inverted "V"		1 [ ]
	spherical		2 [ ]
5.3 (37)	**<u>Only varieties with umbel flower arrangement</u>: Umbel: number of buds**		
	three		1 [ ]
	seven		2 [ ]
	nine		3 [ ]
	eleven		4 [ ]
	> eleven		5 [ ]
5.4 (45)	**Fruit: shape**		
	conical		1 [ ]
	pyriform		2 [ ]
	cylindrical		3 [ ]
	urceolate		4 [ ]
	globose		5 [ ]
	hemispherical		6 [ ]
	campanulate		7 [ ]
	ovoid		8 [ ]

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	Characteristics	Example Varieties	Note
5.5 (49)	**Only for varieties with rhytidome: Trunk: texture of basal rythidome**		
	rough/compact		1 [ ]
	rough/fibrous		2 [ ]
5.6 (?)	**Tree: texture of basal bark on lower part (5 years it is very defined)**		
	rough/compact		1 [ ]
	rough/fibrous		2 [ ]

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6. Similar varieties and differences from these varieties

Please use the following table and box for comments to provide information on how your candidate variety differs from the variety (or varieties) which, to the best of your knowledge, is (or are) most similar. This information may help the examination authority to conduct its examination of distinctness in a more efficient way.

Denomination(s) of variety(ies) similar to your candidate variety	Characteristic(s) in which your candidate variety differs from the similar variety(ies)	Describe the expression of the characteristic(s) for the **similar** variety(ies)	Describe the expression of the characteristic(s) for **your** candidate variety
Example	*[insert example]*	*[insert example]*	*[insert example]*

Comments:

#7. Additional information which may help in the examination of the variety

7.1 In addition to the information provided in sections 5 and 6, are there any additional characteristics which may help to distinguish the variety?

Yes [ ] No [ ]

(If yes, please provide details)

7.2 Are there any special conditions for growing the variety or conducting the examination?

Yes [ ] No [ ]

(If yes, please provide details)

7.3 Other information

Authorities may allow certain of this information to be provided in a confidential section of the Technical Questionnaire.

TECHNICAL QUESTIONNAIRE	Page {x} of {y}	Reference Number:

8. Authorization for release

(a) Does the variety require prior authorization for release under legislation concerning the protection of the environment, human and animal health?

Yes [ ] No [ ]

(b) Has such authorization been obtained?

Yes [ ] No [ ]

If the answer to (b) is yes, please attach a copy of the authorization.

9. Information on plant material to be examined or submitted for examination.

9.1 The expression of a characteristic or several characteristics of a variety may be affected by factors, such as pests and disease, chemical treatment (e.g. growth retardants or pesticides), effects of tissue culture, different rootstocks, scions taken from different growth phases of a tree, etc.

9.2 The plant material should not have undergone any treatment which would affect the expression of the characteristics of the variety, unless the competent authorities allow or request such treatment. If the plant material has undergone such treatment, full details of the treatment must be given. In this respect, please indicate below, to the best of your knowledge, if the plant material to be examined has been subjected to:

(a)	Microorganisms (e.g. virus, bacteria, phytoplasma)	Yes [ ]	No [ ]
(b)	Chemical treatment (e.g. growth retardant, pesticide)	Yes [ ]	No [ ]
(c)	Tissue culture	Yes [ ]	No [ ]
(d)	Other factors	Yes [ ]	No [ ]

Please provide details for where you have indicated “yes”.

………………………………………………………………

10. I hereby declare that, to the best of my knowledge, the information provided in this form is correct:

Applicant’s name

Signature Date

[Annex follows]

ANNEX

Additional Useful Explanations

Part I. Introduction 2

Part II. Characteristics of molecular descriptors 3

Part III. Description of the methods to be used 5

PART I

Introduction

The following Annex contains the characteristics of molecular descriptors to be used for the identification of clones and varieties of *Eucalyptus*. A molecular description has as primary objective, to determine the genetic profile of plants pertaining to the Eucalyptus genus through the analysis of multiple loci in the DNA. Twenty-five loci markers using microsatellite sequences are recommended, and are to be considered as complementary descriptors for the identification of clones, hybrids and varieties of Eucalyptus. The molecular characterization of these loci have been already published in the literature and are being widely used in several laboratories around the world, aiming primarily to the identification of individual trees of *Eucalyptus*, pertaining to almost all the commercially relevant species of sub-genus *Symphyomyrtus*, *Idiogenes* and *Monocalyptus*.

PART II

Characteristics of molecular descriptors

For determination of the genetic profile of a sample, twenty-five microsatellite loci are recommended, according to the table below, to allow a standardization of the genetic profiles generated. At least two molecular markers are listed for each of the eleven linkage groups, corresponding to the eleven chromosomes of *Eucalyptus, but* the analyst can utilize only as many loci as considered necessary for his/her specific situation, looking first for genetically independent markers (in other words, for different linkage groups). However, to allow comparisons among several testing laboratories, it is important for the user to utilize only recommended markers. The higher the number of loci used, the geater the power of discrimination, allowing for more certainty in the identification and comparison process. These loci were published and optimized for genetic identification purposes in *Eucalyptus (Brondani, R.P.V., Brondani, C., Tarchini, R., Grattapaglia, D., 1998. Development and mapping of microsatellite based markers in Eucalyptus. Theoretical and Applied Genetics 97:816-827; Brondani, R.P.V. 2001. Desenvolvimento, caracterização e mapeamento de marcadores microssatélites no gênero Eucalyptus. Tese de doutorado, Biologia Molecular, UnB).*

Table 1

Description of the twenty-five microsatellites markers recommended as molecular descriptors for the genetic profile determination in *Eucalyptus*. The size of the alleles bands located in base of pairs is indicated, as well as sequences of primers and linkage group in the genetic map.

Loci	Allele size (base pairs)	Sequence 5'-3' of direct primer	Sequence 5'-3'of reverse primer	Linkage Group
Embra01	100-145	gatagaactttcctatttgatcg	gtaggatttgatgtctgcaa	8
Embra02	103-148	cgtgacaccaggacattac	acaaatgcaaattcaaatga	11
Embra05	78-142	atgctggtccaactaagatt	tgagcctaaaagcccaac	5
Embra06	120-170	agagaattgctcttcatgga	gaaaagtctgcaaagtctgc	1
Embra10	110-152	gtaaagacatagtgaagacattcc	agacagtacgttctctagctc	10
Embra11	123-165	gcttagaatttgcctaaacc	gtaaaatccatgggcaag	1
Embra12	104-162	aggatttgtggggcaagt	gttccccattttcatgtcc	1
Embra15	90-125	tttgttggatgaggactt	caacatgttctccgaaaag	8
Embra16	110-165	caacgttcccctttcttc	atgttaggccaaacccag	1
Embra17	120-170	aggatactcgtgagagaagc	gtagatctgttctgcatgttg	9
Embra19	55-145	gacggttgatttcctgatt	gtggtgctcctctcctct	4
Embra23	118-145	ggttgtttcatcttttccatg	agcgaaggcaatgtgttt	10
Embra26	112-200	cccacaacaaaaggaaag	agaggtgttcgattcaattc	11
Embra27	100-170	ataaccacaccaatctgca	tatagctcgaacgctcaac	2
Embra28	180-300	caagacatgcatttcgtagt	actcttgatgtgacgagaca	6
Embra34	100-160	tcaaaaccctctctctcat	aataaacattttctttgaacaga	3
Embra37	115-165	cacctctccaaactacacaa	ctcctctctcttcaccattc	5
Embra42	115-170	gagtaaaaattggttttgagtg	ccctcttttcattttgtctt	7
Embra44	205-225	ggggtttgttctgcttag	caaaagagttcagctgtg	4
Embra46	90-130	gaagtcatcatctgtagattgc	acccattattctttgtgagc	7
Embra49	125-195	attattggttcatattgaaaacc	agatagagattgagtgagaccc	3
Embra51	95-200	gatgcattcctttttttcc	cattctcttgcatctggac	6
Embra58	140-245	caccaactggtactatgaggat	ttggcttagggtagaacact	9
Embra63	175-230	catctggagatcgaggaa	gagagaaggatcatgcca	2
Embra72	118-170	ctggtcaacgtccgaaag	atgctgcagagggcataa	10

PART III

Description of the methods to be used

1. Extraction and quantification of DNA: The laboratory will utilize a procedure of extraction and quantification of genomic DNA from plant tissues (leaves, cambium, flowers, etc.). It is suggested that the protocol described by Ferreira & Grattapaglia (*Introdução ao uso de marcadores moleulares em análise genética, 1998. Terceira edição. Embrapa-SPI, pags. 121-130)* be used. The DNA must be quantified by electrophoresis in 0.8% agarose gel followed by ethidium bromide staining.

2. PCR (Polymerase chain reaction): The reactions of PCR for individual loci, are performed with 2 to 50 ng of genomic DNA; 1.5 mM; of Mg++; 0.25 µM of direct and reverse primers; 200 µM of each nucleotide; 0.2 mg/ml BSA; 1 x buffer PCR with 50 mM KCL; 10 mM TRIS-HCL pH 9.0; 0.1% Triton X-100; 1 polymerase unit of *Taq* DNA in a total volume of 15 µl. The PCR program in thermocycler apparatus is composed of an initial denaturation at 95° C for 4 minutes followed by 30 cycles of denaturation at 95° C for 1 minute and an extension at 65° C for 1 minute. There is a final extension step at 65° C for 10 minutes.

3. Polymorphism detection and genotype determination: To have a precise description of genetic profiles, the use of detection systems based upon fluorescence emissions in an automatic DNA sequencer is recommended, which allows for an exact definition of alleles in base pairs with a one base pair resolution. The primers for microsatellite loci must be marked with fluorchromes (blue (FAM); green (HEX); or yellow (NED)) and a specific spectrum filter, according with technology widely used in individual identification in human beings, animals and cultivated plants (*Fregeau, C.J. & Fourney, R.M. 1993 – DNA typing with fluorescently tagged short tamden repeats: a sensitive approach to human identification. Biotechniques 15(1): 100-119)*. Each locus can be analyzed individually, or in "multiplex" combinations for simultaneous analyses of several loci. An internal standard marked with a fluorescent TAMRA or a red color ROX must be used for definition of fragment sizes. The amplified products are spotted on a polyacrylamide gel and separated in an automatic DNA sequencer.

4. Genetic interpretation and communication of descriptors: For each of the analyzed descriptor loci, the observed genotype should be identified and registered. The alleles will be visualized as peaks in the electropherogram and will be identified by their size in base pairs, estimated automatically by using an internal standard of known size (TAMRA or ROX). Genotypes should be described with the alleles identified in number of base pairs, rounded to the unit. The analysis should include, as control check, the DNA of a well characterized *Eucalyptus* clone, to be identified by the laboratory, to serve as a comparison of allele size in base pairs among laboratories or between different experiments within the same laboratory. When considered necessary, the probability of occurrence of the multi loci genetic profile could be estimated, based upon the classic principles of population genetics, assuming a Hardy-Wienberg equilibrium. This probability could be used to

establish significant statistical differences or the genetic identity between two samples, or even the existence of an essential derivation (VED).

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