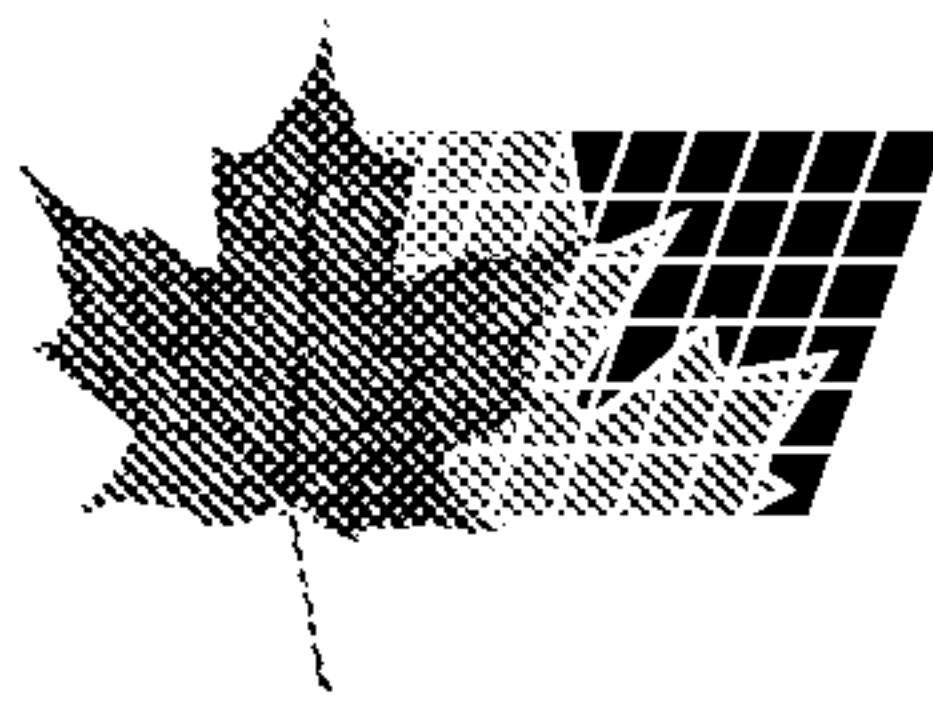




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(54) **LUTTE CONTRE LES MOUSTIQUES ET LES PUCES**

(54) **MOSQUITO AND/OR FLEA CONTROL**



(57) A plant of the species *Leptospermum livensidgei* which is able to block the carbon dioxide message to mosquitoes and/or fleas within the vicinity of its locus. The present invention also provides a crude or refined essential oil extract from the said plant. Another aspect of the invention provides a method for repelling mosquitoes and/or fleas comprising the cultivation of the said plant. A further aspect of the invention provides another method for repelling mosquitoes and/or fleas comprising the use of the said essential oil extract from the said plant.

ABSTRACT

A plant of the species *Leptospermum liversidgei* which is
able to block the carbon dioxide message to mosquitoes
5 and/or fleas within the vicinity of its locus.

The present invention also provides a crude or refined
essential oil extract from the said plant.

10 Another aspect of the invention provides a method for
repelling mosquitoes and/or fleas comprising the
cultivation of the said plant.

A further aspect of the invention provides another method
15 for repelling mosquitoes and/or fleas comprising the use of
the said essential oil extract from the said plant.

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TITLE: MOSQUITO AND/OR FLEA CONTROL

FIELD OF THE INVENTION

5 This invention is in the field of mosquito and/or flea control.

In one aspect the invention relates to a plant which has mosquito and/or flea repellent properties.

10

In another aspect the invention provides an oil extract from the said plant which, in various forms and by various means, may be used as a mosquito and/or flea repellent.

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BACKGROUND TO THE INVENTION

A mosquito's and/or flea's attraction to warm-blooded animals is partly by body warmth and also by carbon dioxide
5 as expelled breath. Certain vaporous substances, such as essential oils, are able to block the carbon dioxide message thus falsely indicating to the mosquito and/or flea that the animal simply is a source of warmth like a light globe or candle.

10

Many species of plants, such as tea-tree (*Leptospermum SP*), are known to produce a large amount of complex mixtures of oils, which are stored in their tissues.

15 It is an object of this invention to isolate and characterise a true-breeding clone of a plant which, by virtue of its oil content, is able to act as a mosquito and/or flea repellent.

20 Such mosquito and/or flea repellency can occur by oxidisation of the oils from the leaves and stems of the plant directly into the surrounding atmosphere. Alternatively, the oils may be extracted from the plant tissue and this extract may be used in aerosol sprays or
25 dispersants or in pharmaceutical preparations suitable for direct topical application to the skin of a human or other warm-blooded animal.

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SUMMARY OF THE INVENTION

The present invention provides:-

- 5 a plant of the Genus *Leptospermum* species *liversidgei* which is able to block the carbon dioxide message to mosquitoes and/or fleas within the vicinity of its locus.

10 The present invention also provides a crude or refined essential oil extract from the said plant.

Another aspect of the invention provides a method for repelling mosquitoes and/or fleas comprising the cultivation of the said plant.

15

A further aspect of the invention provides another method for repelling mosquitoes and/or fleas comprising the use of the said essential oil extract from the said plant.

20 **PREFERRED ASPECTS OF THE INVENTION**

It is advantageous if the plant according to the invention, is aesthetically attractive and thus is suitable as an ornamental plant. The most preferred morphological
25 characteristics are those given in Figures 1 and 2.

The oil which is most suitable for the purpose of the invention is citronellal however the citronellal analogues isopulegol and pulegol are also effective.

30

A plant according to the invention will have a citronellal and/or analogue content which falls substantially within the range of at least 55 - 95% of total extractable volatile oil content. (Throughout this specification, the
35 proportions of oil content are expressed as area percentages measured as areas under peaks by gas-chromatography.)

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The oil content may exhibit seasonal and/or regional variation but it is preferred that it will be at least 70% citronellal and/or analogues.

- 5 This content may be made up of amounts of citronellal, isopulegol and pulegol which fall substantially within the ranges of 59 - 68%, 9 - 14% and 0 - 5% respectively.

10 The most preferred clone of the plant which exhibits the desired morphological features and oil content is genetically characterised by the Randomly Amplified Polymorphic DNA (RAPD) fragment analysis given in Figure 3

15 An essential oil mixture may be extracted from the tissues of the plant according to the invention.

It is preferred that the extracted oil will contain 55 - 95% citronellal and/or the citronellal analogues isopulegol and pulegol. It is more preferred that the oil will
20 comprise at least 70% citronellal and/or analogues.

The most preferred embodiment of this aspect of the invention is an essential oil extract from the plant identified by the RAPD fragment analysis given in Figure 3.

25

The plant according to the invention may be used as a mosquito and/or flea repellent by planting one or more shrubs in either outdoor or indoor situations. The said repellency preferably occurs by oxidation into the
30 surrounding air of oils from the leaves and stems of the plants. Alternatively, the leaves may be manually crushed or bruised to promote the efflux of the oils.

An extracted oil from the plant according to the invention
35 may be used in a method for repelling mosquitoes and/or fleas. The oil may be vaporised by any means known in the art such as a thermal vaporiser (for example an oil burner or other heating device) or by impregnation into a vapour dispersion block or gel.

- 5 -

Alternatively, the oil may be applied directly to the skin or clothing of a human or animal. In this case, it is preferred that the oil is mixed with appropriate pharmaceutical carriers or excipients that make it suitable for topical application. Examples of such preparations include soaps, alcohol-based creams or lotions, oil-based creams and zinc-based creams.

These methods for repelling mosquitoes and/or fleas may be used individually or in combination with each other.

BRIEF DESCRIPTION OF THE FIGURES AND TABLES

Figure 1 is a photograph of the most preferred clone of *Leptospermum liversidgei* according to the invention.

Figure 2 is a sketch of the same plant depicted in Figure 1 with colours of the various parts identified by RHS Colour Coding Standards.

Figure 3 shows the results of Randomly Amplified Polymorphic DNA (RAPD) fragment analysis of the plant shown in Figure 1.

Table 1 characterises the DNA restriction fragments that were used as standards of known size for comparison with the RAPD fragments in Figure 3

Table 2 shows the results of gas chromatographic analysis of ethanolic extracts of leaf tissue from various individual plants according to the invention.

Table 3 gives details of the analysis parameters for the gas chromatographic mass spectrometric analysis of

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essential oil extracts.

Table 4 shows the results of gas chromatographic mass spectrometric analysis of oils extracted from the most preferred clone of the plant according to the invention,

Table 5 shows the nucleotide sequences of the primers used to generate RAPD fragments, and

Table 6 sets out the cycling conditions used in the PCR amplification of DNA for RAPD analysis.

DETAILED DESCRIPTION WITH RESPECT TO THE FIGURES AND TABLES

15 Morphology

Leptospermum liversidgei Baker & Smith.

See Figures 1 and 2.

20 Shrub, erect, compact, 0.5 - 2.5 (3) metres tall and 1-1.5 metres wide, mostly single trunked; branches numerous, erect, ends occasionally lightly weeping, sericeous, soon becoming glabrous.

25 Bark hard, tight, grey, flaking in patches near base.

Leaves shortly petiolate, alternate, densely arranged up the stems, seldom spreading, flexible, apex obtuse, 3-8mm long x 1.5-3.5mm wide, glabrous, new growth RHS Green group 141C; mature leaves RHS Green group 141A, oil glands small, numerous, emitting a citrus-like scent when crushed.

Flowers solitary, ± sessile, mainly in axils; hypanthium 3-5mm long x 3-4mm wide, glabrous or occasionally sparsely sericeous; sepals ca 1.5mm long; petals light pink RHS Red group 56D, ca 5-7mm long x 4-5mm wide; stamens numerous C.15-20; ovary five locular, domed, glabrous.

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Propagation of plant

Following clonal selection on the basis of tissue oil content, the plant has been propagated asexually through
5 three generations using standard nursery techniques.

These include material cleansing, hormone stimulation, and root generation on heat beds in a glasshouse situation in a medium comprised of equal parts of peat and grade 500
10 perlite.

Growing-on has been carried out in a soil-less potting medium, comprising milled pinebark and coarse sand, with added trace elements and NPK fertiliser regime supplied by
15 controlled release pellets.

Extraction and Analysis of Essential Oil Content.

Leaf tissue was obtained in June from various individuals
20 of *Leptospermum liversidgei*. Ethanolic extracts of each sample were analysed by the single leaf extract method published by Brophy et al, the disclosure of which is incorporated herein by reference. (J J Brophy, N W Davies, I A Southwell, I A Skiff and L R Williams, Journal of
25 Agricultural and Food Chemistry 1989, 37 1330-1335). Analysis was by a 60mm x 0.2mmid AT35 FSOT gas chromatography column using hydrogen as a carrier gas.

Results of this analysis are shown in Table 2. The sample
30 with the highest total citronellal and analogue level was No. BY11 with a figure of 73%.

A further sample of this most preferred individual was taken from cultivated material when the plant was
35 vigorously growing in March. The following procedure was used to distil the essential oils from this material.

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Leaf tissue was placed in a steam distillation vessel with some boiling chips. The vessel was then filled with water to cover the plant material. The distillation cover was securely attached with a wire clamp. A Dean and Stark
5 apparatus and water cooled condenser were then attached using a stand and clamps for support. A hot plate was placed under the distillation vessel and adjusted to bring the contents to the boil.

10 The essential oil was removed from the plant material by the steam, which travelled upwards to the condenser where it cooled into liquid form, collecting in the Dean and Stark apparatus. The oil was the lower layer in the apparatus and was removed via the tap. The oil was then
15 dried for analysis.

Analysis of the oil content was by gas chromatography and mass-spectrometry, by the parameters outlined in Table 3. As shown in Table 4, this analysis was able to identify
20 pulegol as well as the isopulegol and citronellal identified earlier. The measurement of total citronellal/analogue content of the oil had increased by this method to 82.5%.

25 Genetic characterisation of the selected clone.

Genetic characterisation of BY11 was undertaken by generation of a Randomly Amplified Polymorphic DNA (RAPD) profile.

30

Firstly, DNA was isolated from leaf tissue of BY11 according to the method described by Fulton et al (T M Fulton, J Chunwongse and S D Tanksley, Plant Molecular Biology Reporter 1995 13, 207-209) the entire disclosure of
35 which is incorporated herein by reference. The only modification to this method was the addition of 2% soluble polyvinyl pyrrolidone (PVP) to the micropreparation buffer.

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RAPD fragments were then generated from aliquots of this DNA by the following protocol:-

Twenty separate PCR DNA amplification reactions were
5 carried out, each with a different primer (2-01 to 2-20,
the sequences of which are shown in Table 5) under the
cycling conditions set out in Table 6. The reaction
mixtures contained: DNA, 50ng; Primer, 0.4 μ M; dNTPs, 100 μ M;
Mg²⁺, 3 μ M; X1 buffer; Amplitaq, 1.0 Unit; in a total
10 reaction volume of 50 μ l. When the reactions were
complete, 20 μ l aliquots of reaction product were sampled
from each 50 μ l reaction mixture and added to gel loading
buffer. These samples were loaded onto a 1% agarose gel in
X 1 TAE buffer and the gel was electrophoretically
15 developed at 70V which separated the DNA fragments
according to size. For comparison as known size standards,
two separate preparations of Hind III and SPP1 restriction
fragments of λ DNA (as shown in Table 1) were also loaded
into the 1% agarose gel in an amount of 0.5 μ g total DNA
20 per lane. The resulting ethidium bromide stained fragments
were visualised with UV light and recorded photographically
as shown in Figure 3.

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TABLE 1

SPP1 = Standard A (S_A)

Fragment No	Size (Kb) mean $\pm$ SD**	Molecular Weight*
1	8.51 $\pm$ 0.07	5,62 $\times 10^6$
2	7.35 $\pm$ 0.06	4.85 $\times 10^6$
3	6.11 $\pm$ 0.06	4.03 $\times 10^6$
4	4.84 $\pm$ 0.03	3.19 $\times 10^6$
5	3.59 $\pm$ 0.01	2.37 $\times 10^6$
6	2.81 $\pm$ 0.01	1.85 $\times 10^6$
8	1.86 $\pm$ 0.02	1.23 $\times 10^6$
9	1.51 $\pm$ 0.01	0.99 $\times 10^6$
10	1.39 $\pm$ 0.02	0.92 $\times 10^6$
11	1.16 $\pm$ 0.01	0.76 $\times 10^6$
12	0.98 $\pm$ 0.01	0.64 $\times 10^6$
13	0.72 $\pm$ 0.00	0.47 $\times 10^6$
14	0.48 $\pm$ 0.01	0.32 $\times 10^6$
15	0.36 $\pm$ 0.01	0.24 $\times 10^6$

 λ Hind III = Standard B (S_B)

Fragment No	No of Base Pairs	Molecular Weight
1	23,130	15.26 $\times 10^6$
2	9,416	6.21 $\times 10^6$
3	6,557	4.32 $\times 10^6$
4	4,361	2.88 $\times 10^6$
5	2,322	1.53 $\times 10^6$
6	2,027	1.33 $\times 10^6$
7	564	0.37 $\times 10^6$
8	125	0.08 $\times 10^6$

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TABLE 2

Sample No	pinene	linalool	citronellal	iso-pulegol	un-identified (1)	un-identified (2)	B-caryophyllene	un-identified (3)
BY11	0.6	1.5	59.2	13.8	4.0	8.6	1.4	4.5
13	8.1	2.6	48.0	12.0	2.5	6.1	2.9	4.8
16	1.2	1.2	50.5	10.9	2.9	11.2	1.4	3.4
BY18	0.9	1.6	52.2	12.5	2.2	8.4	1.3	5.2
25	1.8	2.8	49.5	9.4	1.9	11.3	2.5	3.3

TABLE 3

Instrument	Varian GC 3400/Saturn MS
Autosampler	Varian 8100
Column	Dual J&W fused silica capillary, 0.25 micron DB5 coating.
Column Size	0.26 mm i.d. x 30m
Sample size	0.1 microlitres of 4% solution in absolute alcohol
Split ratio	20 to 1
Carrier gas	Helium @ 60 psi, 1 ml/min
Make up gas	Nitrogen @ 40 psi
Injector temp	250 deg C
GC Detector	FID A
Detector temp	250 deg C
Oven temp initial	60 deg C
Oven temp final	240 deg C
Oven temp program rate	3 deg/min from 60 - 240 deg C
Final temp hold time	2 min
FID Autozero	On
FID Attenuation	8
FID Range	12
Plot speed	0.3 cm/min
Zero offset	15%
Peak reject value	30
Signal to noise ratio	5
Tangent peak height	5
Initial peak width	2
Audiliary temp	240 deg C
Manifold temp	170 deg C
Multiplier set voltage	1700 volts
A/M amplitude set voltage	3.5 volts
Low mass	41 m/z
High mass	320 m/z
Scan rate	1000 millisecs
Segment acquire time	62 min
Peak threshold	1 count
Filament/multiplier delay	2.00 min
Mass defect	100 m μ /100 μ
Tune file	TUN_DFLT
Emission current	20 microamps
AGC prescan ionisation time	100 microsecs
AGC prescan storage level	20.0 m/z
El background mass	45 m/z
RF dump value	650.0 m/z
AGC target	32500 counts
El max ionisation time	25000 microsecs

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TABLE 4

BY11

alpha Pinene	0.7%
beta Pinene	0.1
Myrcene	0.1
Limonene +	
1,8 Cincol	0.1
Linalool	1.4
iso Pulegol	9.7
Citronellal	67.9
Pulegol	4.9
nCO Menthol	0.6
Citonellol	1.4
Noral	0.2
Geraniol	0.2
Methyl citronellate	0.1
Geranial	0.2
Citronellyl acetate	5.7
t Methyl cinnamate +	
Geranyl acetate	1.9
beta Caryophyllene	1.3
alpha Humulene	0.1
Germacrene D	0.1
Germacrene B	1.1
Ledol	0.1
Spathulenol	0.1
Globulol	0.5
beta Eudesmol	0.1
	95.6% identified

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TABLE 5

Sequence of primers

Primers	Sequence 5'-----3'	Primers	Sequence 5'-----3'
2-01	AAGCTGCGAG	2-11	CCGAATTCCC
2-02	CACGGCGAGT	2-12	GGCTGCAGAA
2-03	CTGGCGTGAC	2-13	CTGACCAGCC
2-04	GGGTAACGCC	2-14	GTCCCGTGGT
2-05	CAATCGCCGT	2-15	CCACACTACC
2-06	TTCGAGCCAG	2-16	CACCCGGATG
2-07	GAACGGACTC	2-17	CACAGGCGGA
2-08	GTCCCGACGA	2-18	TGACCCGCCT
2-09	TGTCATCCCC	2-19	GGACGGCGTT
2-10	GGTGATCAGG	2-20	TGGCGCAGTG

TABLE 6

Cycle Number	Temperature (°C)	Time	Step in Cycle
1	94	1 min	denaturation
	40	30 sec	annealing
	72	1 min	extension
2-34	94	10 sec	denaturation
	40	30 sec	annealing
	72	1 min	extension
35	94	10 sec	denaturation
	40	30 sec	annealing
	72	5 min	extension
	4	Hold	

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The claims, illustrations, photographs and drawings, if any, form part of the disclosure of this specification as does the description, claims, illustrations, photographs and drawings of any associated provisional or parent
5 specification or of any priority document, if any, all of which are imported hereinto as part of the record hereof.

Finally it is to be understood that various alterations, modifications and/or additions may be incorporated into the
10 various constructions and arrangements or parts without departing from the spirit and ambit of the invention.

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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A plant of the Genus *Leptospermum* species
5 *liversidgei*, which is able to block the carbon dioxide message to mosquitoes and/or fleas within the vicinity of its locus.
2. A plant as claimed in Claim 1, which has the
10 morphological characteristics given in Figure 1 and Figure 2.
3. A plant as claimed in Claim 1 or Claim 2, which has
a citronellal and/or analogue content which falls
15 substantially within the range of at least 55 - 95% of total extractable volatile oil content.
4. A plant as claimed in Claim 3, wherein the plant is
genetically characterised by the Randomly Amplified
20 Polymorphic DNA (RAPD) fragment analysis given in Figure 3.
5. A plant substantially as hereinbefore described with reference to any one of the accompanying drawings.

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6. An essential oil extract derived from a plant as claimed in Claim 4.
7. An essential oil extract, which contains 55 - 95% citronellal and/or the citronellal analogues isopulegol and pulegol.
8. An essential oil extract, which is from the plant identified by the RAPD fragment analysis given in Figure 3.
9. A mosquito and/or flea repellent comprising the essential oil of Claims 6, 7 or 14.
10. An essential oil substantially as hereinbefore described with reference to any one of the accompanying drawings.
11. An essential oil extract derived from a plant of the Genus *Leptospermum* species *liversidgei*, in which the plant is able to block the carbon dioxide message to mosquitoes and/or fleas within the vicinity of its locus.

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12. An essential oil as claimed in Claim 11, in which the plant has the morphological characteristics given in Figure 1 and Figure 2.
- 5 13. An essential oil as claimed in Claim 11 or Claim 12, in which the plant has a citronellal and/or analogue content which falls substantially within the range of at least 55 - 95% of total extractable volatile oil content.
- 10 14. An essential oil as claimed in Claim 13, in which the plant is genetically characterised by the Randomly Amplified Polymorphic DNA (RAPD) fragment analysis given in Figure 3.
- 15 15. An essential oil extract derived from a plant substantially as hereinbefore described with reference to any one of the accompanying drawings.
- 20 16. A method for repelling mosquitoes and/or fleas comprising cultivation of a plant of the Genus *Leptospermum* species *liversidgei* which is able to block the carbon dioxide message to mosquitoes and/or fleas within the vicinity of its locus.

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17. A method as claimed in Claim 16, in which the plant has the morphological characteristics given in Figure 1 and Figure 2.

5 18. A method as claimed in Claim 16 or 17, in which the plant has a citronellal and/or analogue content which falls substantially within the range of at least 55 - 95% of total extractable volatile oil content.

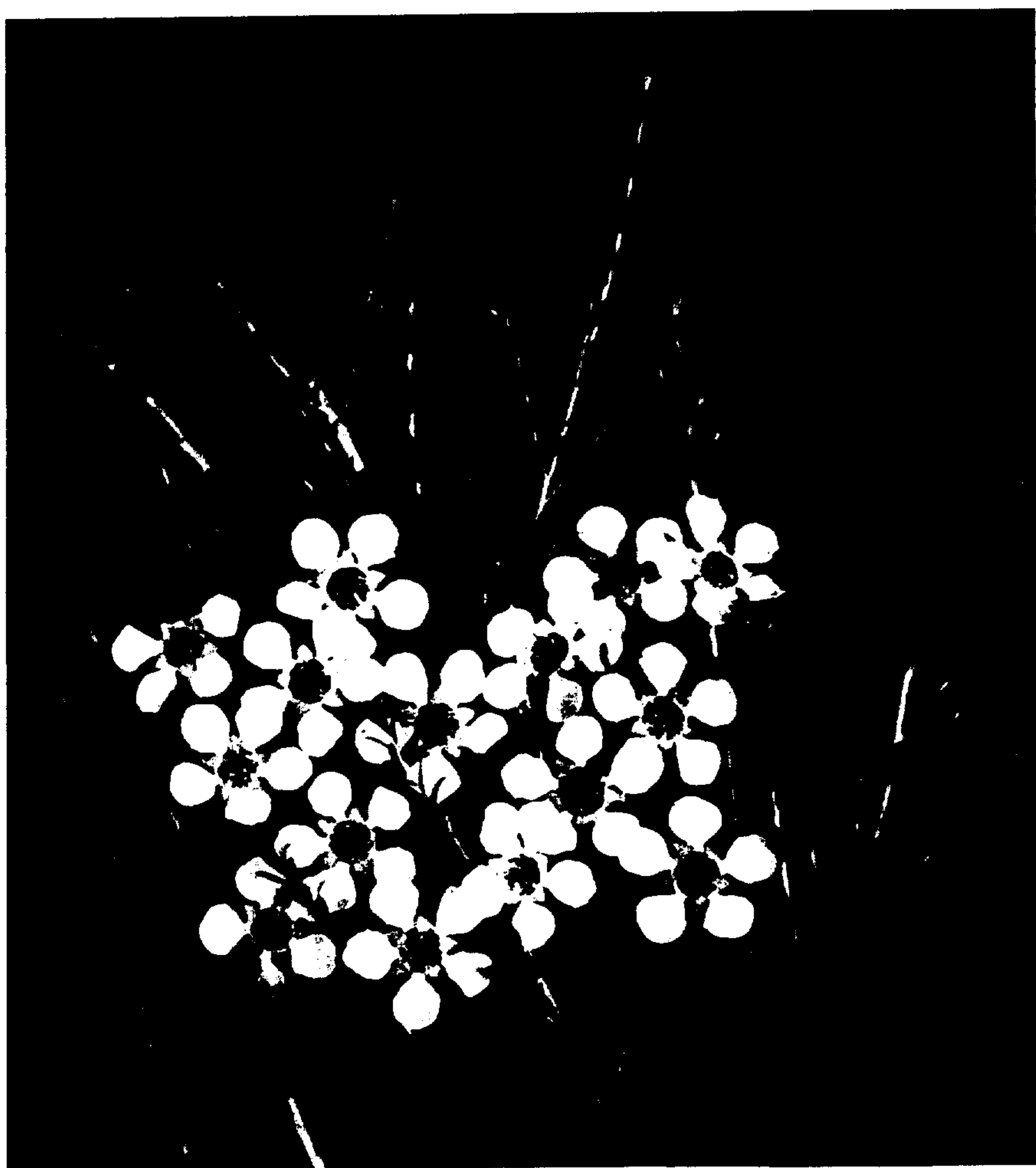
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19. A method as claimed in Claim 18, in which the plant is genetically characterised by the Randomly Amplified Polymorphic DNA (RAPD) fragment analysis given in Figure 3.

15

20. A method for repelling mosquitoes and/or fleas comprising the use of an essential oil extract as claimed in Claims 9 or 14.

Fig. 1



Colour coding R.H.S. standards

bud: CALYX LOBES green 50C

Petals Red group 56B

Opening flowers: petals red group 56C

Mature flowers: petals red group 56D

CALYX: Top Yellow/green 149B

Edge: 56B

Figure 2

BAR SCALE = 1 cm.



Leptospermum
liversidgei (BAKER & SMITH)
'Mottie Blocker'

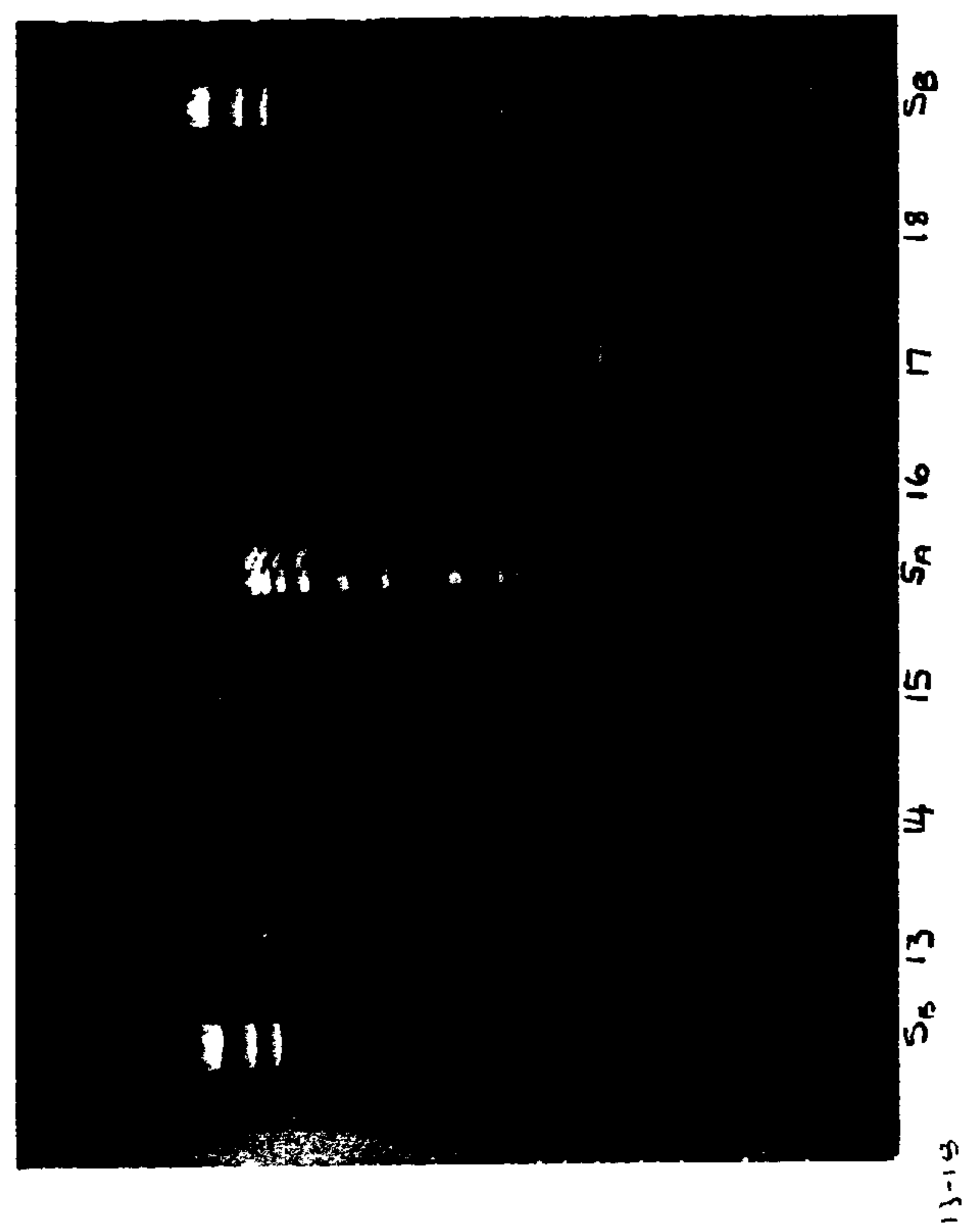
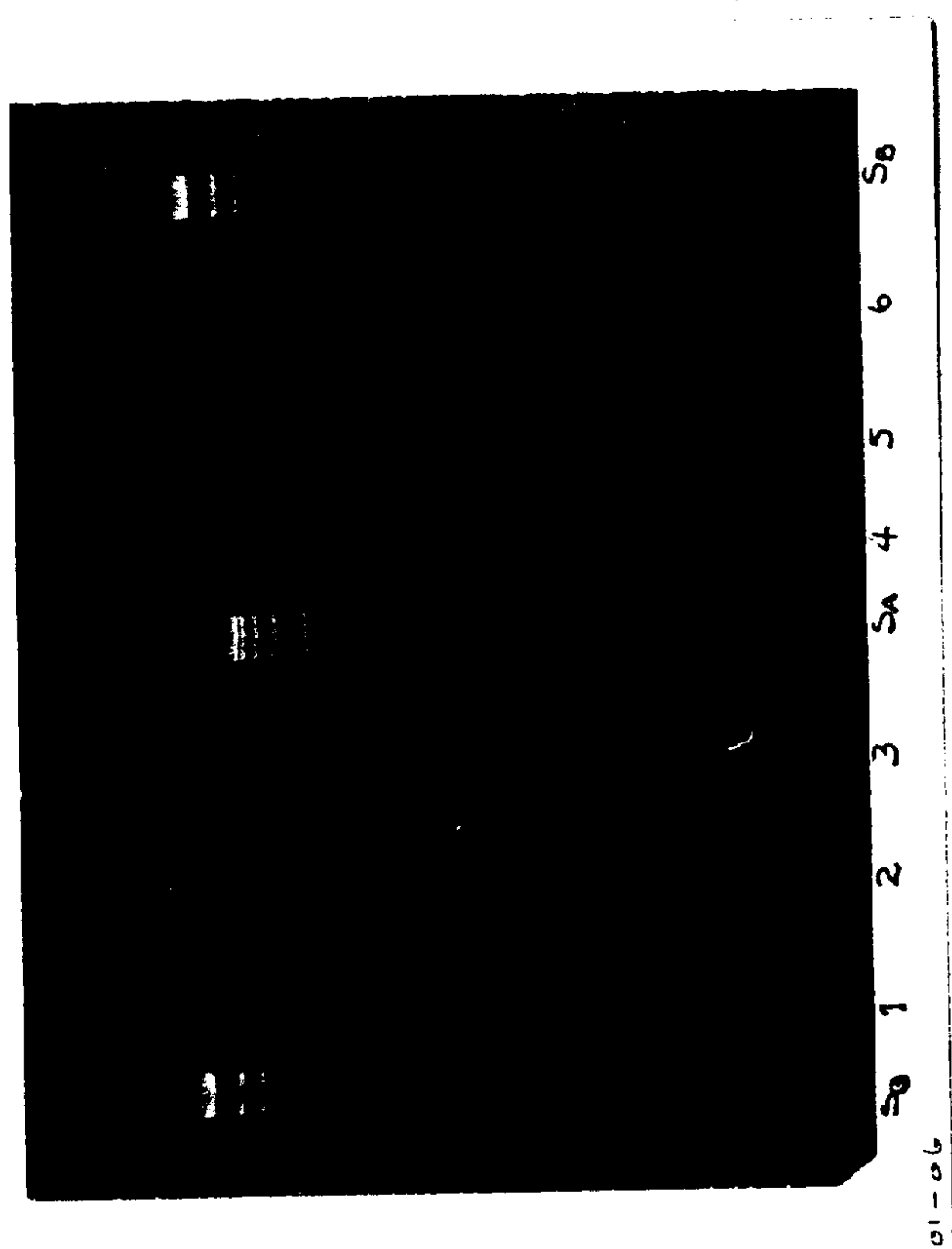
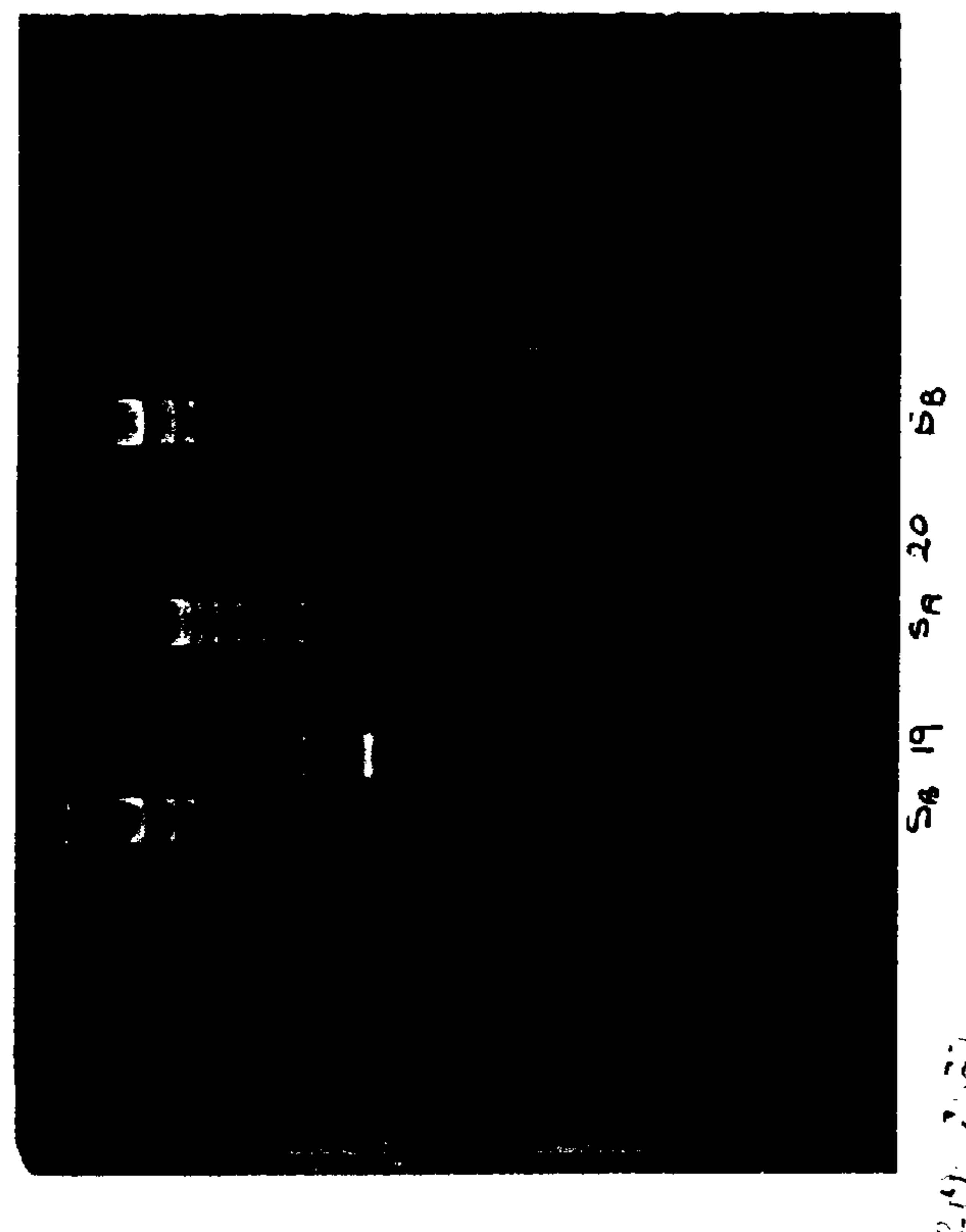
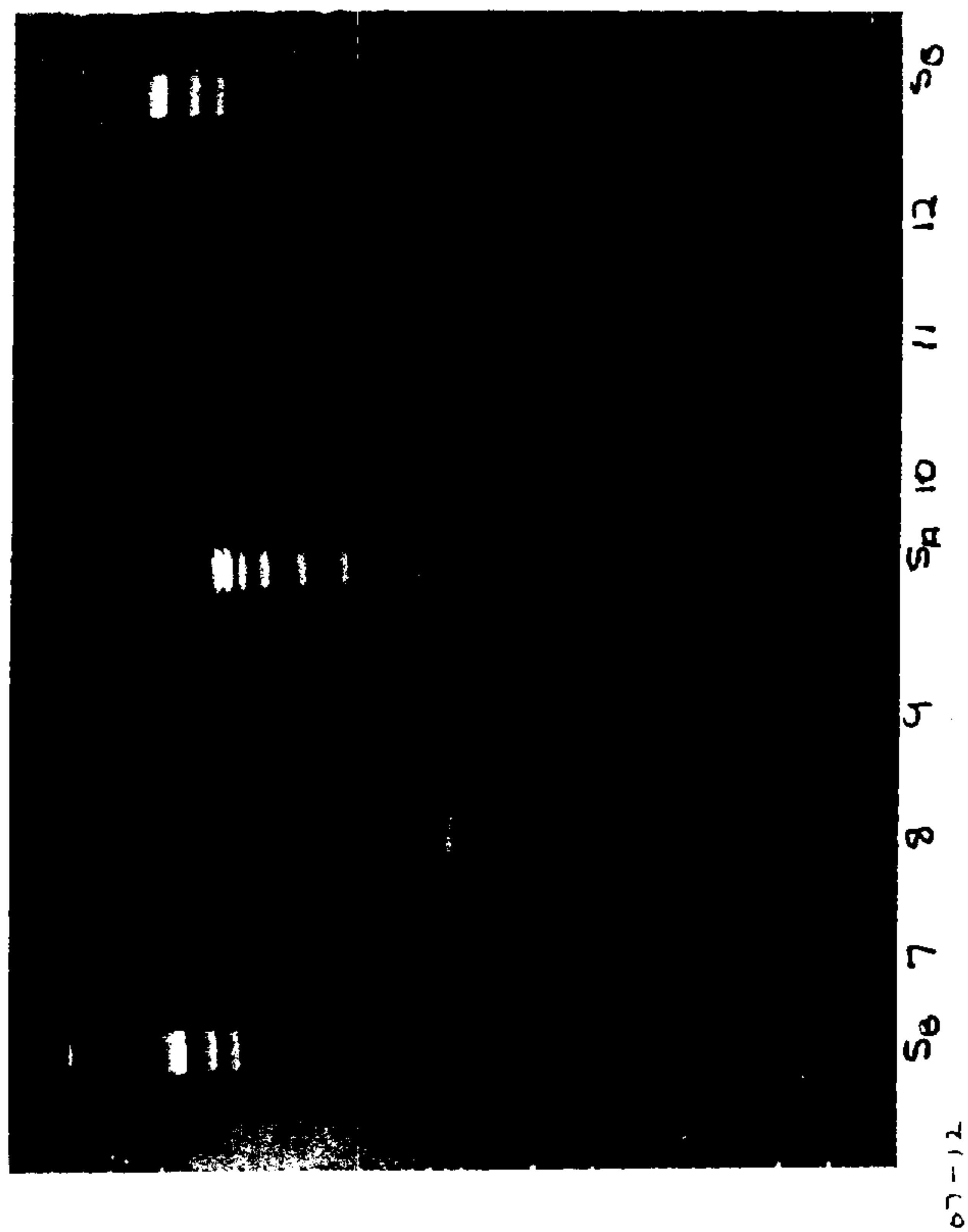


Figure 3 -

RAPD fragments of BY11 using primers 2.01 - 2.20 as indicated. Fragments of standard size were included on each gel and are indicated as S_A or S_B . (See Table 1)