



16S rRNA Analizi

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**Ankara Üniversitesi Tıp Fakültesi
Tıbbi Mikrobiyoloji Anabilim Dalı**



Sunum içeriđi

- Genel bilgi
- Uygulanışı
- Kullanım alanları
- Avantajları
- Dezavantajları



Neden moleküler (genetik) tanı?

- **Fenotipik identifikasyon**
 - **Uzun zaman alır**
 - 24-48 saat
 - Bazen günler, haftalar, aylar...
 - **Bazen sonuç vermez**
 - Atipik biyokimyasal özellikler
 - Biyokimyasal olarak inaktivite
 - Özel üretim şartlarına ihtiyaç
 - Laboratuvar ortamında üretilememe



Hedef Genler

- Fonksiyonu evrim boyunca değişmemiş olmalı
- Tüm bakteriler için korunmuş bir bölgeye sahip olmalı
- Korunmuş bölgeyi çevreleyen ileri derecede değişken bölgeler bulunmalı

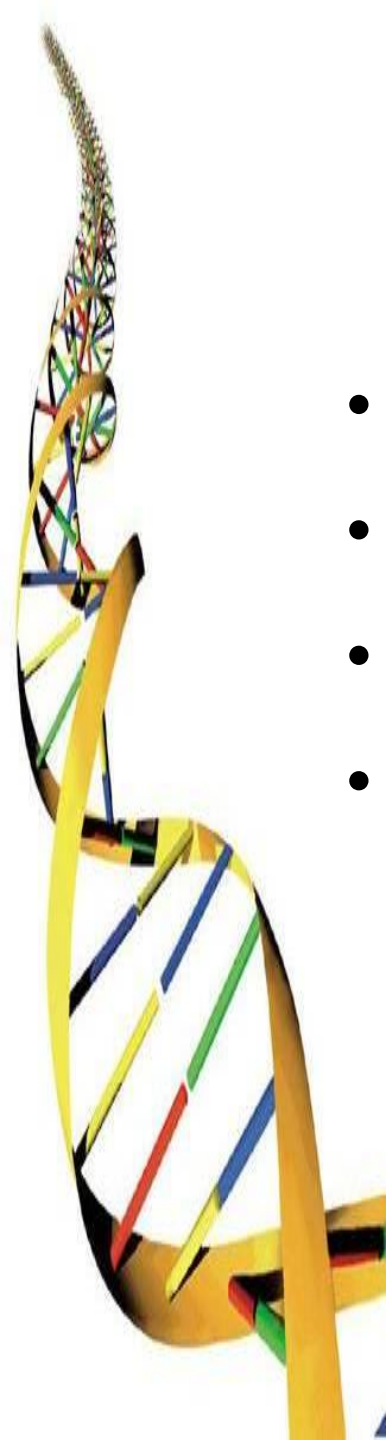


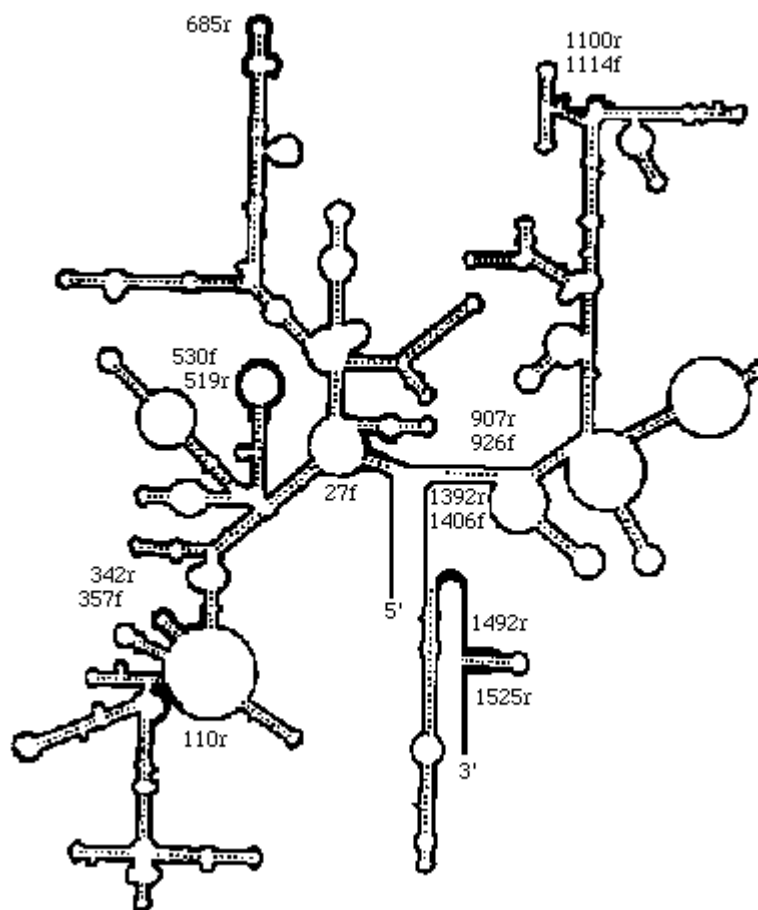
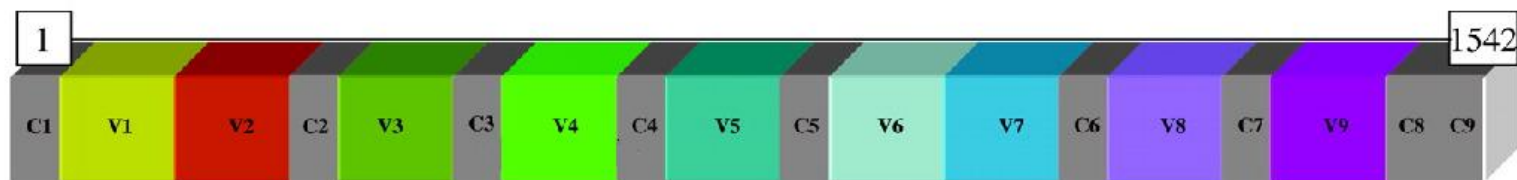
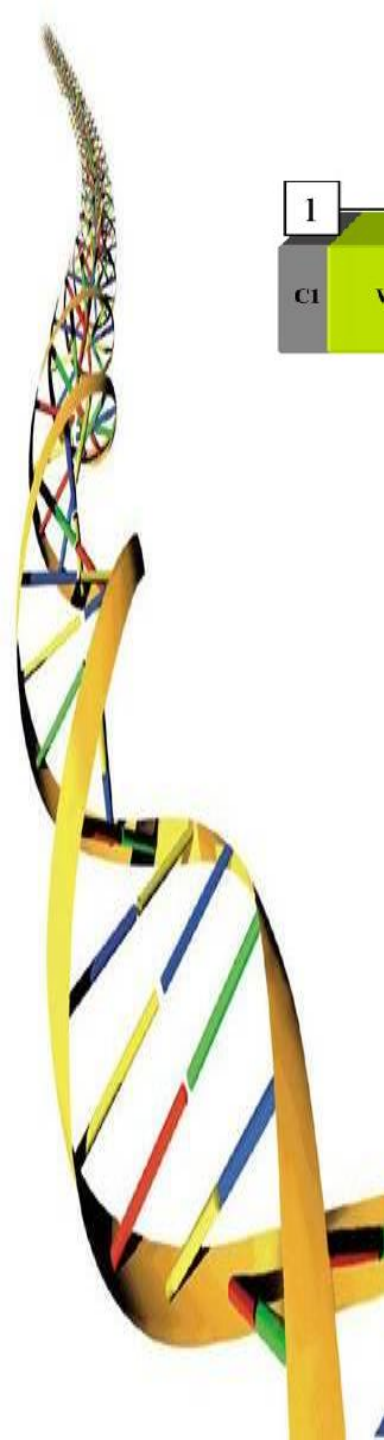
Hedef Genler

- 
- **16S rRNA**
 - **23S rRNA**
 - **16S-23S rRNA ITS**
 - ***groEL***
 - ***rpoB***
 - ***recA***
 - ***gyrA***
 - ***gyrB***
 - ***hsp65***
 - ***tuf***
 - ***sodA***
 - ***ospA***
 - ***fla***

Neden 16S rRNA Geni?

- **Bütün bakterilerde bulunur**
- **Fonksiyonel olarak korunmuştur**
- **Uygun büyüklüktedir (~1550 bç)**
- **Üzerinde iyi korunmuş ve heterojen bölgeler içerir**
 - **PZR primerleri oluşturulabilir**
 - **Cins/tür düzeyinde ayrım sağlar**





Neden 16S rRNA Geni?

- Yeterli veritabanı vardır

Gen Bölgesi

- 16S rRNA
- 23S rRNA
- 16S-23S rRNA ITS
- *groEL*
- *rpoB*
- *recA*
- *gyrB*

Kayıtlı Dizi Sayısı

2.344.816

45.121

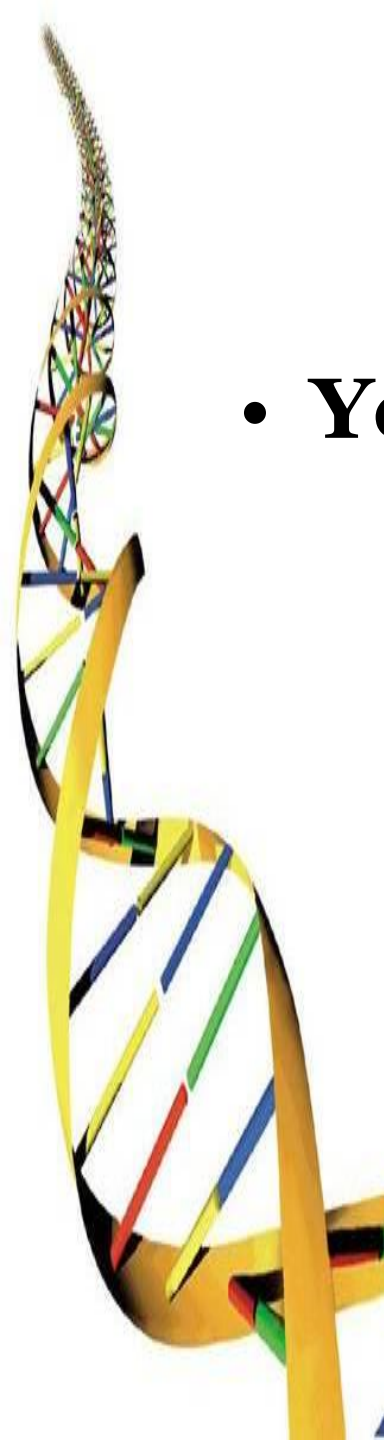
27.445

14.478

23.532

18.662

17.410



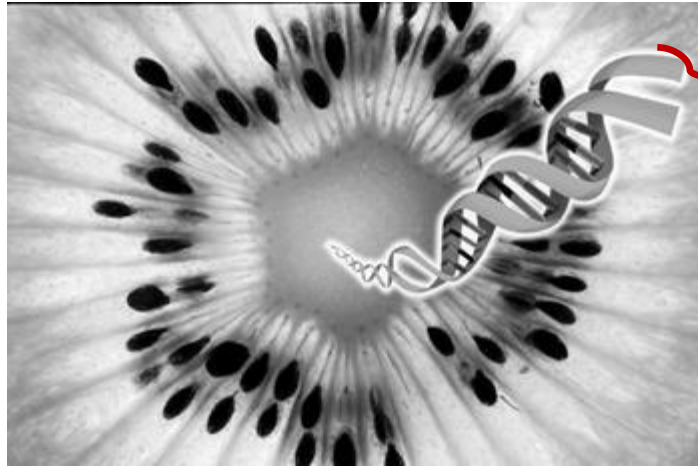
Nasıl Uygulanır?

1. **DNA Ekstraksiyonu**
2. **16S rRNA PZR**
3. **Pürifikasyon**
4. **Sekans döngüsü**
5. **Pürifikasyon**
6. **Sekans analizi**
7. **Elektroferogramların değerlendirilmesi**
8. **Verilerin yorumlanması**



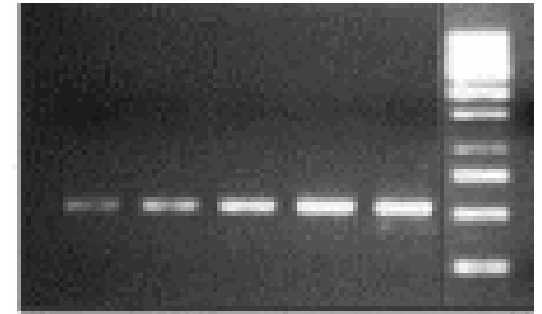
1. DNA Ekstraksiyonu

- Klinik örnekten
- Kültür kolonisinden
- Basit yöntemler tercih edilmeli



2. 16S rRNA PZR

- ~ 500 bç
 - Sıklıkla yeterli
- ~ 1500 bç
 - Birden fazla sekans analizi
- < 500 bç
 - DNA'nın hasarlı olma ihtimali varsa



Kullanılan primerler

İsim	Dizi (5'-3')	Lokalizasyon
27f	AGA GTT TGA TYM TGG CTC AG	8-27
355f	ACT CCT ACG GGA GGC AGC	338-355
338r	GCT GCC TCC CGT AGG AGT	338-355
533f	GTG CCA GCM GCC GCG GTA A	515-533
515r	TTA CCG CGG CKG CTG GCA C	515-533
556r	CTT TAC GCC CAR TRA WTC CG	556-575
806f	ATT AGA TAC CCT GGT AGT CC	787-806
787r	GGA GTA CCA GGG TAT CTA AT	787-806
926f	AAA CTY AAA KGA ATT GAC CG	907-926
907r	CCG TCA ATT CMT TTR AGT TT	907-926
930f	AAA CTY AAA KGA ATT GAC GG	911-930
911r	CCG TCA ATT CMT TTR AGT TT	911-930
1194f	GAG GAA GGT TGG GGA TGA CGT	1175-1194
1175r	ACG TCA TCC CCA ACC TTC CTC	1175-1194
1371r	AGG CCC GGG AAC GTA TTC AC	1390-1371
1391r	TGA CGG GCG GTG WGT RCA	1391-1408
1492r	GGT TAC CTT GTT ACG ACT T	1492-1510
1525r	AAG GAG GTG WTC CAR CC	1525-1541

Y: C/T

R: G/A

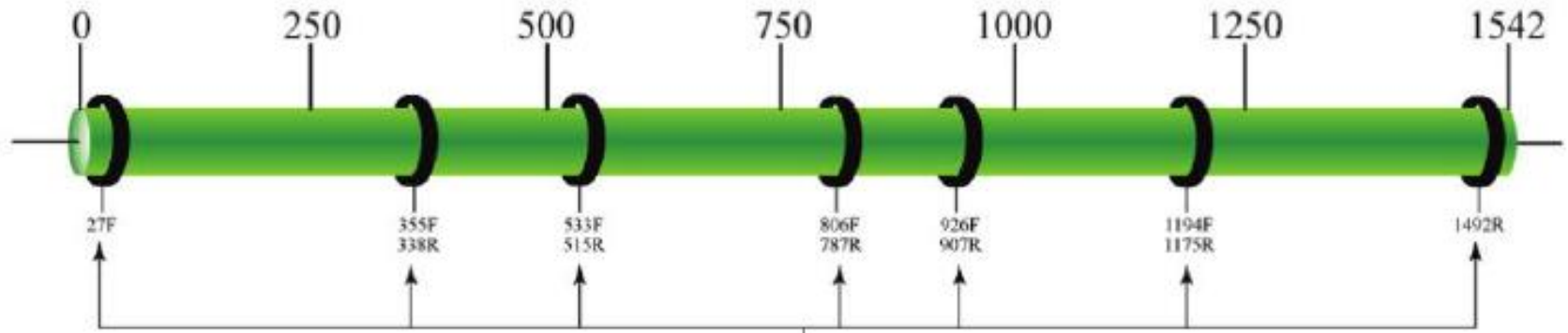
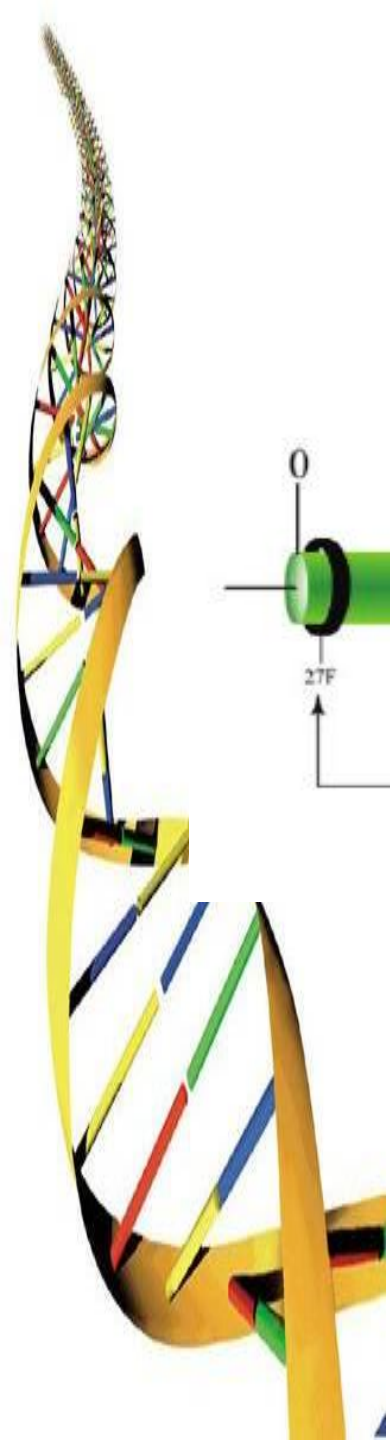
M: A/C

K: G/T

W: A/T

S: G/C

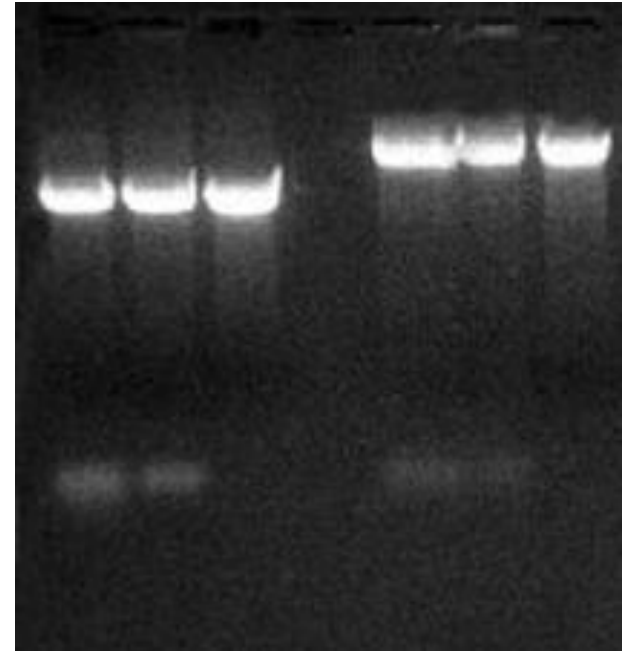




Primerlerin yerleştiği korunmuş bölgeler

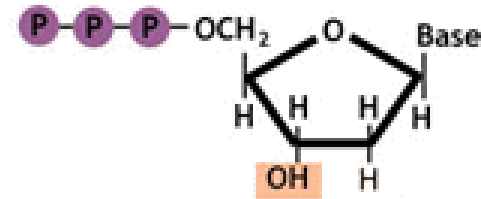
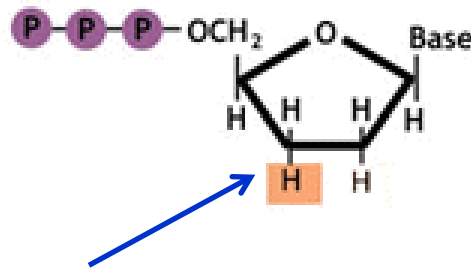
3. Pürifikasyon

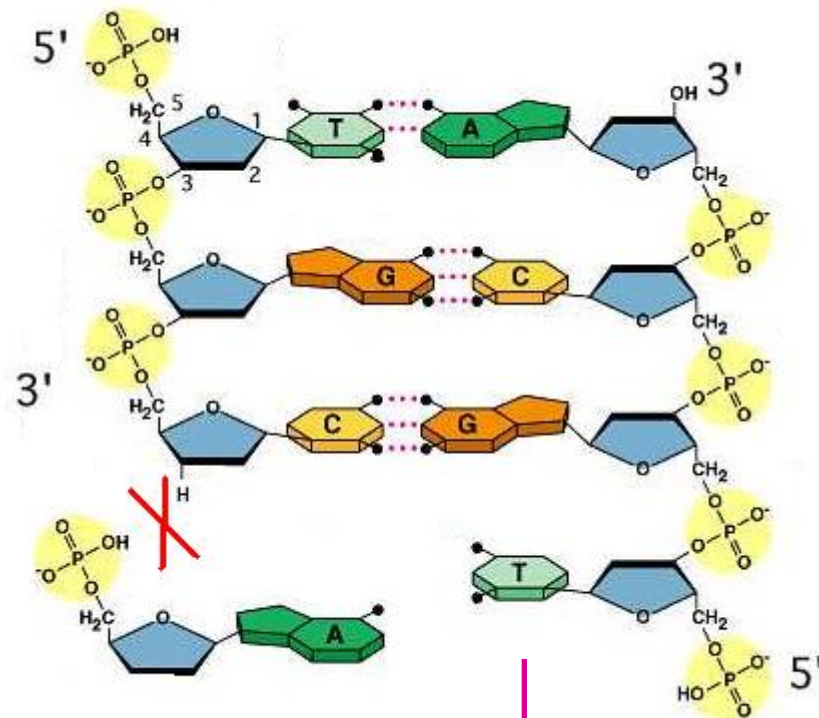
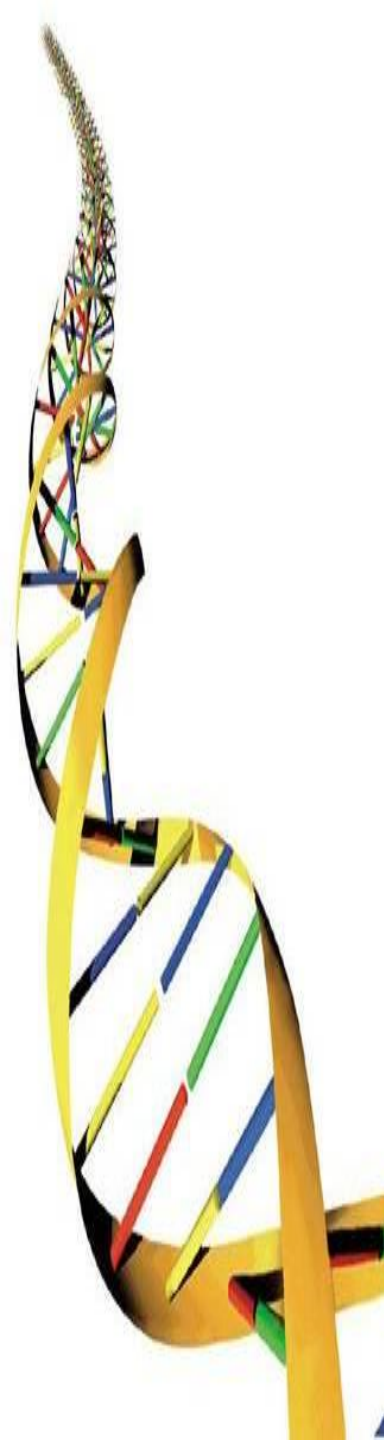
- Standart yöntemler
- Ticari kitler
- Jelden
- PZR ürününden



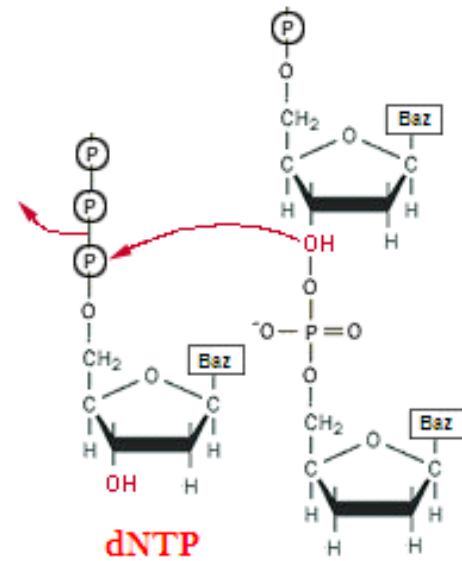
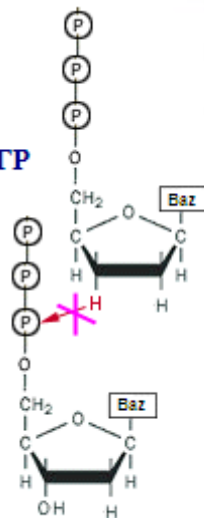
4. Sekanslama Döngüsü ("Cycle sequencing")

- Yaklaşık 25 döngü
- Yeni PZR ürünü oluşmaz
- Farklı uzunluklarda ürünler oluşur
 - Tek primer
 - dNTP
 - Sonlandırıcı boyalar (Farklı dalga boyunda floresans veren boyalarla işaretli ddNTP)





ddNTP



CA
CAG
CAGT
CAGTC
CAGTCG
CAGTCGG
CAGTCGGT
CAGTCGGTA

5. Pürifikasyon

- Standart yöntemler
- Ticari kitler



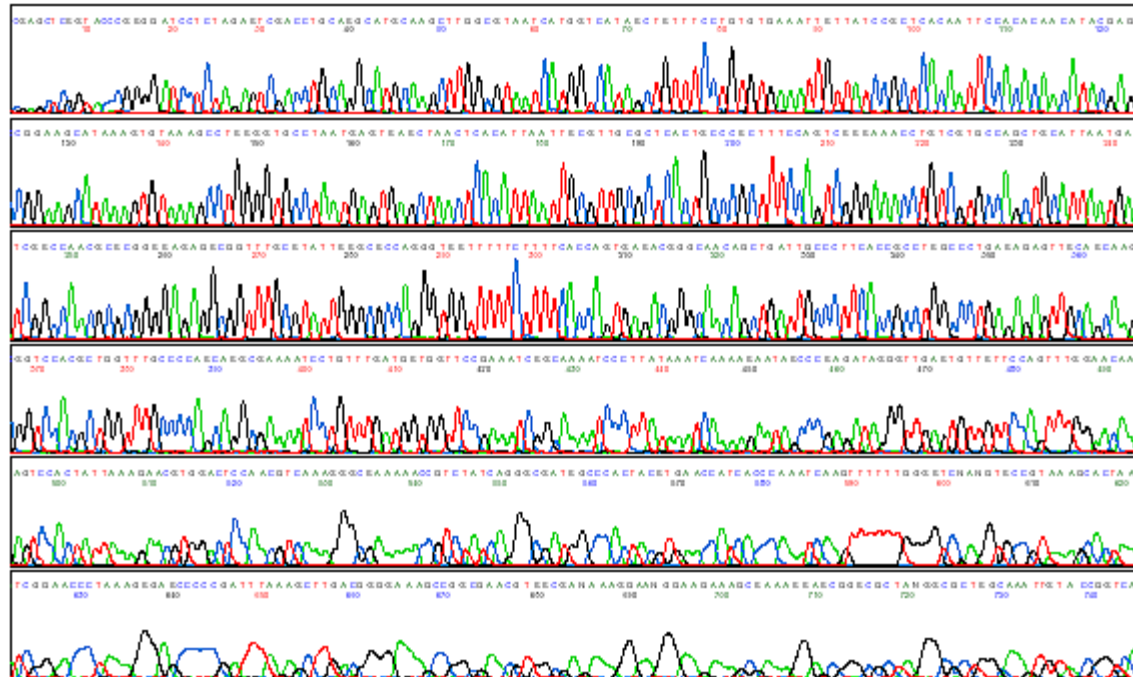
6. Sekans Analizi

- Kapiller elektroforez sistemli otomatik sekans cihazları
- Sanger yöntemiyle çalışır
- Sonlandırıcı boyaları okur
- Elektroferogram oluşturur



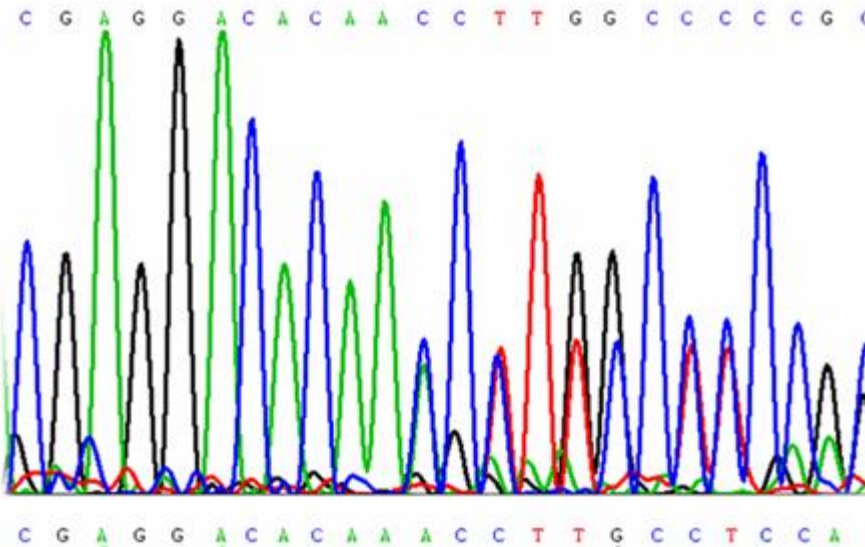
ddNTP **Dalga boyu** **Floresans** **Pik rengi**

G	540 nm	yeşil	siyah
A	570 nm	yeşil/sarı	yeşil
T	595 nm	kırmızı	kırmızı
C	620 nm	turuncu/kırmızı	mavi



7. Elektroferogramların Değerlendirilmesi

- Otomatik rapor
- Gözle değerlendir!!!
- Karşılaştır



Veri Tabanlarıyla Karşılaştırma

- GenBank

<http://blast.ncbi.nlm.nih.gov/Blast.cgi>

- RDP-II

http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp

- Ticari

MicroSeq 500 (Applied biosystems)

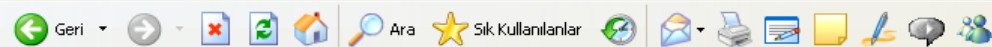
500 bç kütüphane

1818 tür/alt tür

Tüm 16S rRNA geni

1396 tür/alt tür



Adres <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

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- ☐ [Danio rerio](#)
- ☐ [Drosophila melanogaster](#)
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Choose a BLAST program to run.

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Algorithms: [blastp](#), [psi-blast](#), [phi-blast](#)[blastx](#)Search **protein** database using a **translated nucleotide** query[tblastn](#)Search **translated nucleotide** database using a **protein** query[tblastx](#)Search **translated nucleotide** database using a **translated nucleotide** query

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- ☐ Search [trace archives](#)
- ☐ Find [conserved domains](#) in your sequence (cds)

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[BLAST 2.2.24 release](#)

A new version of the stand-alone applications is available.

Mon, 23 Aug 2010 13:00:00 EST

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Enter Query Sequence

BLASTn programs search nucleotide databases using a nucleotide query. [more...](#)[Reset page](#)[Bookmark](#)Enter accession number, gi, or FASTA sequence [Clear](#)Query subrange 

From

To

Or, upload file

 Gözet...

Job Title

Enter a descriptive title for your BLAST search ☐ Align two or more sequences 

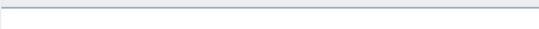
Choose Search Set

Database

☐ Human genomic + transcript ☐ Mouse genomic + transcript ☒ Others (nr etc.):Nucleotide collection (nr/nt)  

Organism

Optional

 ☐ Exclude  

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

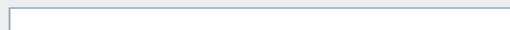
Exclude

Optional

☐ Models (XM/XP) ☐ Uncultured/environmental sample sequences

Entrez Query

Optional

Enter an Entrez query to limit search

Program Selection

Optimize for

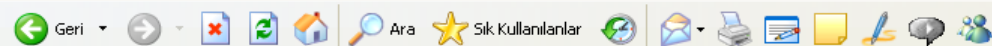
- ☒ Highly similar sequences (megablast)
☐ More dissimilar sequences (discontiguous megablast)
☐ Somewhat similar sequences (blastn)

Choose a BLAST algorithm 

BLAST

Search database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)

☐ Show results in a new window

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Enter Query Sequence

Enter accession number, qi, or FASTA sequence ?

Clear

Query subrange

ctttctgattaggtaccgtcaagacgtgcatagttacttacacatttgtcttccctaataacagagtttta
cgatccgaagaccttcatactcacgcggcggtgtgctcogtcaggcttctgccattgcggaagattccctac
tgctgcctcccgtagagagcttggaacctgtctcaagtccagtgctgccgacacctctcaggctcggtacg
catcgctcttcttggaagccgttacctaccaactcgaataatgcggcgcggtatccatctataagtgacagca
aaacgcctctccctattgaaacctgcagctccggttcaatata

From To

Or, upload file

Gözet...

Job Title

Enter a descriptive title for your BLAST search

☐ Align two or more sequences

Choose Search Set

Database

☐ Human genomic + transcript ☐ Mouse genomic + transcript ☒ Others (nr etc.):

Nucleotide collection (nr/nt)

Organism

Optional

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Exclude

Optional

☐ Models (XM/XP) ☐ Uncultured/environmental sample sequences

Entrez Query

Optional

Enter an Entrez query to limit search 

Program Selection

Optimize for

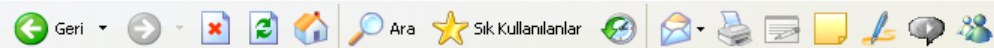
- ☒ Highly similar sequences (megablast)
- ☐ More dissimilar sequences (discontiguous megablast)
- ☐ Somewhat similar sequences (blastn)

Choose a BLAST algorithm

BLAST

Search **database Nucleotide collection (nr/nt)** using **Megablast (Optimize for highly similar sequences)**

☐ **Check results in a new window**

Adres <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

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Job Title: Nucleotide Sequence (326 letters)

Request ID	D57W2DKT011
Status	Searching
Submitted at	Thu Nov 4 07:56:50 2010
Current time	Thu Nov 4 07:56:55 2010
Time since submission	00:00:04

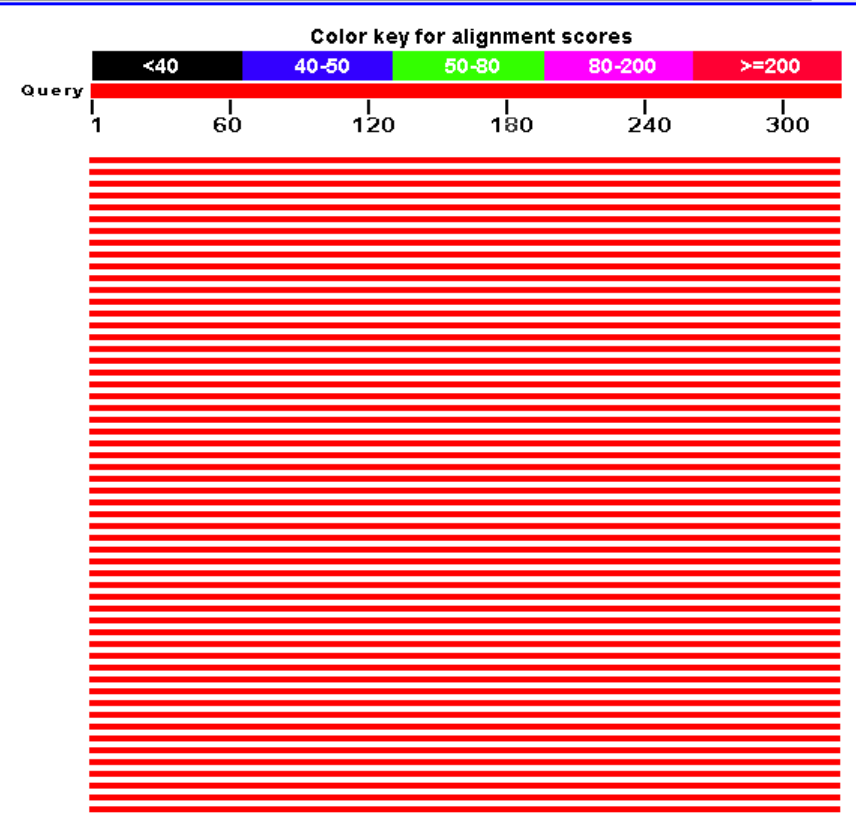
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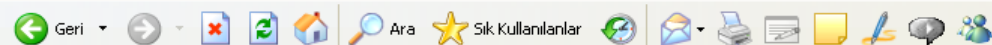
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Distribution of 100 Blast Hits on the Query Sequence

Mouse-over to show define and scores, click to show alignments



Adres <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

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Tablolar
BioAssay

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
GU980229.1	Staphylococcus epidermidis strain CCGE4064 16S ribosomal RNA qen	603	603	100%	3e-169	100%	
HQ202847.1	Staphylococcus epidermidis strain T-3 16S ribosomal RNA gene, part	603	603	100%	3e-169	100%	
HQ154577.1	Staphylococcus epidermidis strain R7-1A 16S ribosomal RNA gene, p.	603	603	100%	3e-169	100%	
HM218641.1	Staphylococcus epidermidis strain NM160-2 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM218511.1	Staphylococcus epidermidis strain NM127-2 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM218371.1	Staphylococcus epidermidis strain NM88-2 16S ribosomal RNA gene,	603	603	100%	3e-169	100%	
HM218280.1	Staphylococcus epidermidis strain NM62-4 16S ribosomal RNA gene,	603	603	100%	3e-169	100%	
HM218008.1	Staphylococcus epidermidis strain MGB83-2 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM217991.1	Staphylococcus epidermidis strain S45-2 16S ribosomal RNA gene, p.	603	603	100%	3e-169	100%	
HM217990.1	Staphylococcus epidermidis strain S45-1 16S ribosomal RNA gene, p.	603	603	100%	3e-169	100%	
HM217960.1	Staphylococcus epidermidis strain G58-1 16S ribosomal RNA gene, p.	603	603	100%	3e-169	100%	
HM217957.1	Staphylococcus epidermidis strain G55-1 16S ribosomal RNA gene, p.	603	603	100%	3e-169	100%	
HQ148903.1	Uncultured Staphylococcus sp. clone SF42 16S ribosomal RNA gene,	603	603	100%	3e-169	100%	
GQ476207.1	Uncultured bacterium clone 1703-M13F 16S ribosomal RNA gene, par	603	603	100%	3e-169	100%	
HQ011917.1	Staphylococcus epidermidis strain U11 16S ribosomal RNA gene, part	603	603	100%	3e-169	100%	
FR666717.1	Staphylococcus epidermidis partial 16S rRNA gene, isolate TKU3a	603	603	100%	3e-169	100%	
HM163562.1	Staphylococcus epidermidis strain 1DL27M1a 16S ribosomal RNA qen	603	603	100%	3e-169	100%	
HM163540.1	Staphylococcus epidermidis strain 4TT12 16S ribosomal RNA gene, p	603	603	100%	3e-169	100%	
HM163539.1	Staphylococcus epidermidis strain 4TT11 16S ribosomal RNA gene, p	603	603	100%	3e-169	100%	
HM163537.1	Staphylococcus epidermidis strain 4TL13 16S ribosomal RNA gene, p.	603	603	100%	3e-169	100%	
HM163527.1	Staphylococcus epidermidis strain 4DT214M 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM163500.1	Staphylococcus epidermidis strain 3DL257M 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM163493.1	Staphylococcus epidermidis strain 3DL134M 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM163481.1	Staphylococcus epidermidis strain 2DT127M 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM163479.1	Staphylococcus epidermidis strain 2DL287MA 16S ribosomal RNA qen	603	603	100%	3e-169	100%	
HM163478.1	Staphylococcus epidermidis strain 2DL244M 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM163475.1	Staphylococcus epidermidis strain 2DL64M 16S ribosomal RNA gene,	603	603	100%	3e-169	100%	
HM163474.1	Bacillus endophyticus strain 2DL47M 16S ribosomal RNA gene, partial	603	603	100%	3e-169	100%	
HM163472.1	Staphylococcus epidermidis strain 2DL114M 16S ribosomal RNA gene	603	603	100%	3e-169	100%	
HM216570.1	Staphylococcus epidermidis strain m030608b 16S ribosomal RNA qen	603	603	100%	3e-169	100%	
HM077180.1	Uncultured Staphylococcus sp. clone VA21_89 16S ribosomal RNA qe	603	603	100%	3e-169	100%	
HM077168.1	Uncultured Staphylococcus sp. clone VA21_77 16S ribosomal RNA qe	603	603	100%	3e-169	100%	
HM077131.1	Uncultured Staphylococcus sp. clone VA21_39 16S ribosomal RNA qe	603	603	100%	3e-169	100%	
HM077008.1	Uncultured Staphylococcus sp. clone VA19_94 16S ribosomal RNA qe	603	603	100%	3e-169	100%	
HM076990.1	Uncultured Staphylococcus sp. clone VA19_75 16S ribosomal RNA qe	603	603	100%	3e-169	100%	
HM076985.1	Uncultured Staphylococcus sp. clone VA19_69 16S ribosomal RNA qe	603	603	100%	3e-169	100%	
HM076979.1	Uncultured Staphylococcus sp. clone VA19_63 16S ribosomal RNA qe	603	603	100%	3e-169	100%	
HM076952.1	Uncultured Staphylococcus sp. clone VA19_32 16S ribosomal RNA qe	603	603	100%	3e-169	100%	

[Distance](#)
[tree](#)
[of](#)
[results](#)

> [gb|GU980229.1](#) Staphylococcus epidermidis strain CCGE4064 16S ribosomal RNA gene, partial sequence
Length=1390

Score = 603 bits (326), Expect = 3e-169
Identities = 326/326 (100%), Gaps = 0/326 (0%)
Strand=Plus/Minus

```
Query 1   CTTTCTGATTAGGTACCGTCAAGACGTGCATAGTTACTTACACATTTGTTCTTCCCTAAT 60
          |||
Sbjct 420 CTTTCTGATTAGGTACCGTCAAGACGTGCATAGTTACTTACACATTTGTTCTTCCCTAAT 361

Query 61  AACAGAGTTTTACGATCCGAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTT 120
          |||
Sbjct 360 AACAGAGTTTTACGATCCGAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTT 301

Query 121 CGCCCATTCGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGCTCTCAGT 180
          |||
Sbjct 300 CGCCCATTCGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGCTCTCAGT 241

Query 181 TCCAGTGTGGCCGATCACCTCTCAGGTGCGCTACGCATCGTTGCTTGGTAAGCCGTTA 240
          |||
Sbjct 240 TCCAGTGTGGCCGATCACCTCTCAGGTGCGCTACGCATCGTTGCTTGGTAAGCCGTTA 181

Query 241 CCTTACCAACTAGCTAATGCGGCGCGGATCCATCTATAAGTGACAGCAAAACCGTCTTTC 300
          |||
Sbjct 180 CCTTACCAACTAGCTAATGCGGCGCGGATCCATCTATAAGTGACAGCAAAACCGTCTTTC 121

Query 301 ACTATTGAACCATGCGGTTCAATATA 326
          |||
Sbjct 120 ACTATTGAACCATGCGGTTCAATATA 95
```

> [gb|HQ202847.1](#) Staphylococcus epidermidis strain T-3 16S ribosomal RNA gene, partial sequence
Length=1455

Score = 603 bits (326), Expect = 3e-169
Identities = 326/326 (100%), Gaps = 0/326 (0%)
Strand=Plus/Minus

```
Query 1   CTTTCTGATTAGGTACCGTCAAGACGTGCATAGTTACTTACACATTTGTTCTTCCCTAAT 60
          |||
Sbjct 472 CTTTCTGATTAGGTACCGTCAAGACGTGCATAGTTACTTACACATTTGTTCTTCCCTAAT 413

Query 61  AACAGAGTTTTACGATCCGAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTT 120
          |||
Sbjct 412 AACAGAGTTTTACGATCCGAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTT 353

Query 121 CGCCCATTCGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGCTCTCAGT 180
          |||
Sbjct 352 CGCCCATTCGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGCTCTCAGT 293

Query 181 TCCAGTGTGGCCGATCACCTCTCAGGTGCGCTACGCATCGTTGCTTGGTAAGCCGTTA 240
          |||
Sbjct 292 TCCAGTGTGGCCGATCACCTCTCAGGTGCGCTACGCATCGTTGCTTGGTAAGCCGTTA 233
```



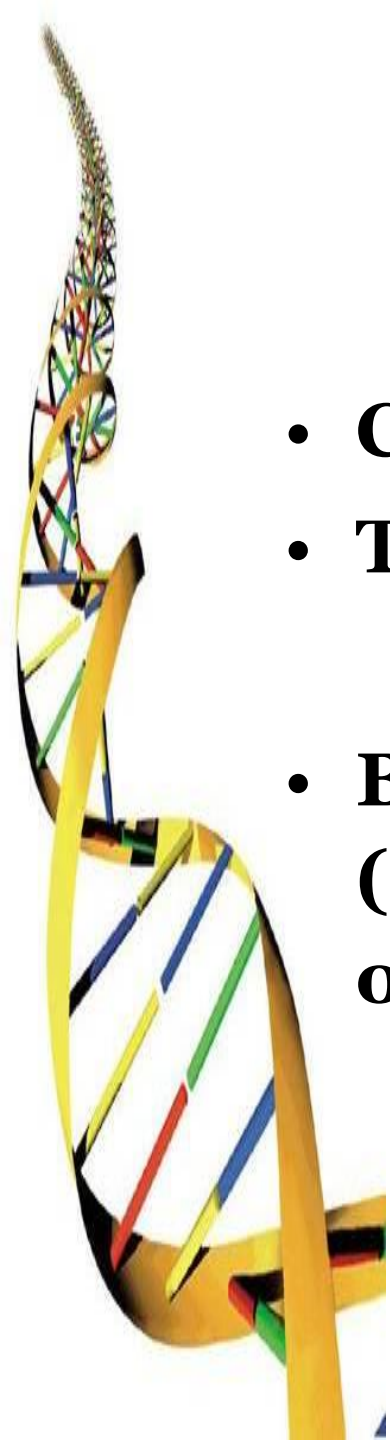
Eşleşen nükleotid sayısı
Total nükleotid sayısı

8. Verilerin Yorumlanması ve İsimlendirme



Cins? Tür?

- CİNS : $\geq \%97$ dizi benzerliği
- TÜR : $\geq \%99$ dizi benzerliği
- Bu oranlar mikroorganizma türüne (genetik heterojenite oranına) bağlı olarak değişiklik gösterebilir





Problemler

– Türler arası yüksek dizi benzerliği

- Bazı *Mycobacterium* türleri (%100 benzerlik)
- Bazı *Bacillus* türleri (>%99.5 benzerlik)
- *Edwardsiella* türleri (%99.35-99.81)
- *Streptococcus mitis/oralis/pneumoniae* (>%99)

– İntragenomik heterojenite

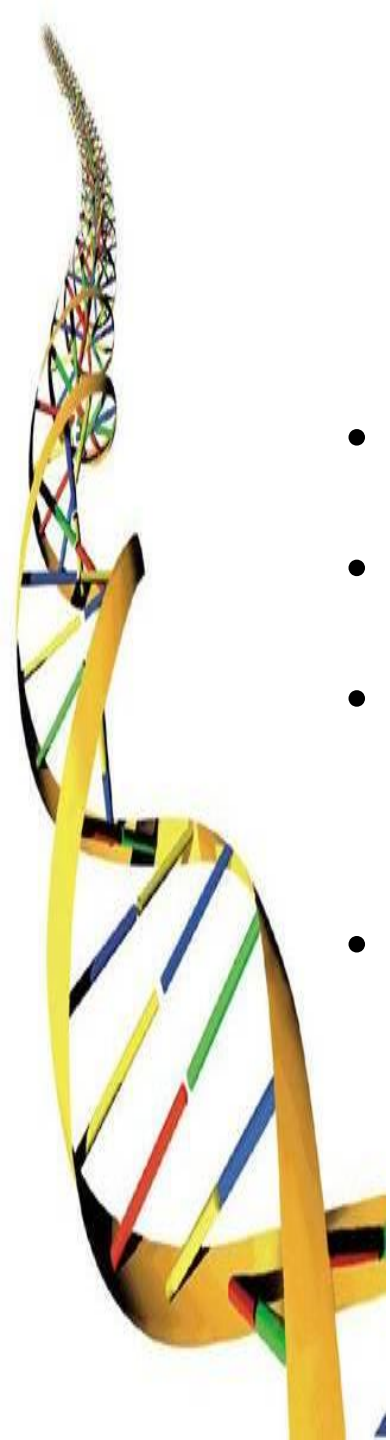
- *A. veronii*: %1.5

– Tür düzeyinde sorun yaşanan diğer bakteriler:

- *Achromobacter* spp., *Actinomyces* spp., *Aeromonas* spp., *A. baumannii-calcoaceticus* kompleksi, *Bordetella* spp., *Burkholderia* spp., *Campylobacter* spp., *Enterobacter* spp., *Neisseria* spp., *Pseudomonas* spp., *Stenotrophomonas* spp.,

Ne Yapmalı?

- **En yakın cins nedir?**
- **O cinsten 16S rRNA değışkenliđi nedir?**
- **En fazla benzerlik taşıyan dizi ile bir sonraki seçenek arasında fark?**
- **Bu cinsin bütün türleri veri tabanında yer alıyor mu?**



Ne Yapmalı?

- Tüm dizi sekanslama?
- Bir başka hedef gen?
- Ek biyokimyasal testler?
- Yeni tür?
 - Tüm dizide $> \%1$ dizi farkı
 - Tüm dizide $\leq \%1$ dizi farkı + fenotipik özgünlük



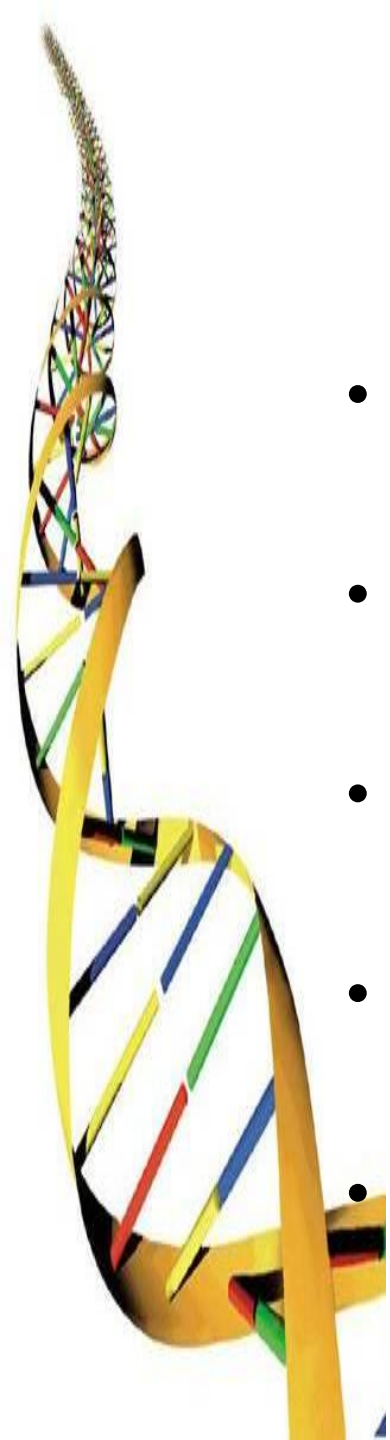
Kullanım Alanları

- Yeni patojenlerin keşfi
 - *Bartonella henselae*
 - *Tropheryma whipplei*
 - *Mycobacterium genavense*
 - Moleküler Koch Postülatları !



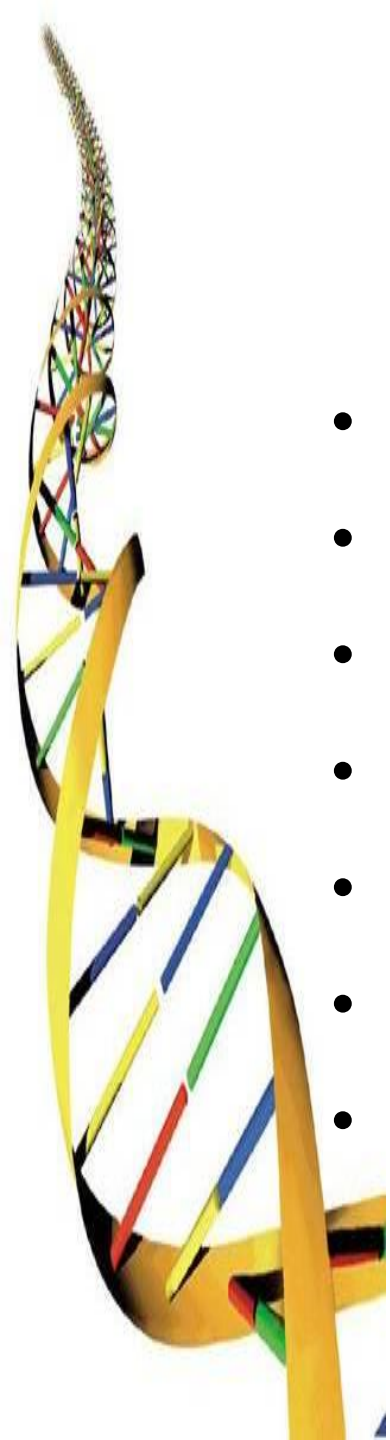
Kullanım Alanları

- **Taksonomik güncellemeler, yeni cins ve türlerin belirlenmesi**
- **Kültürde üretilemeyen/ zor üreyen mikroorganizmaların tanısı**
- **Klinik önemi olan patojenlerin tanımlanması**
- **Antibiyotik tedavisi altında etken belirlenmesi**
- **“Steril” örneklerde etkenin hızlı belirlenmesi**



Avantajları

- **Seçici değildir**
- **Hızlıdır**
- **Sonuçları net ve kesindir**
- **Antibiyotik tedavisi altında kullanılabilir**
- **Klinik örnekten direkt çalışılabilir**
- **Sonuçlar arşivlenebilir**
- **Epidemiyolojik çalışmalar için uygundur**



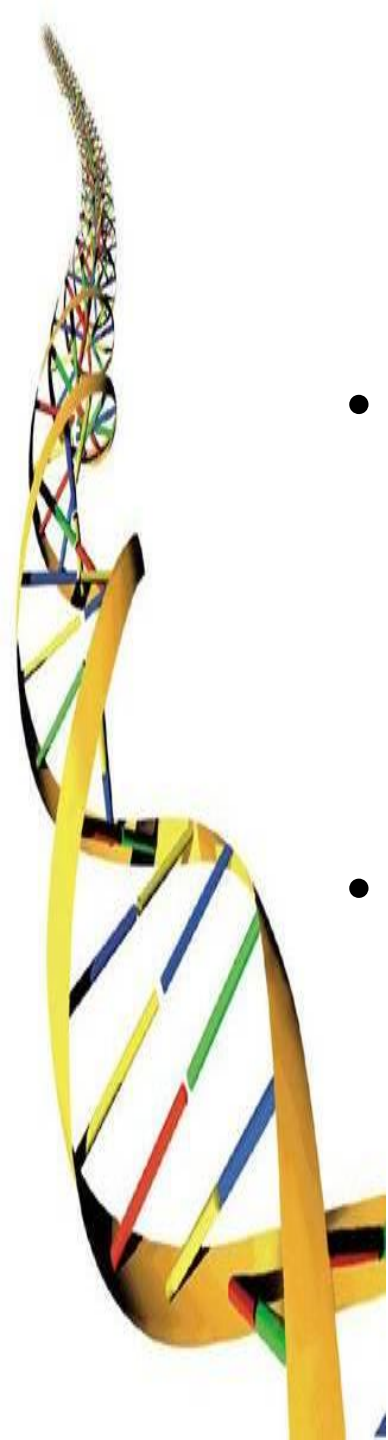
Dezavantajları

- **Pahalıdır**
- **Teknik donanım gerektirir**
- **Deneyimli personel gerektirir**
- **Dikkatli uygulama gerektirir**
- **Bazı tür/alt türler için tanımlayıcılığı azdır**
 - Tür içi değişkenliğin az olması
 - Tek türe ait çok sayıda genom var bulunması
 - Veritabanında yer almaması
 - Doğrulayıcı biyokimyasal testlerin olmaması
 - Taksonomik değişiklikler



Dezavantajları

- **Uygulama ve yorumlamada standart metodoloji ve kılavuzlar yoktur**
 - Cins/tür tanımı tartışmalıdır
- **İdeal bir veritabanı yoktur**
 - Kullanıma açık olmalı
 - Yeterli sayıda suşla temsil edilmeli
 - Denetlenmeli
 - Güncellenmeli



Sonuç

**Rutin mikrobiyoloji laboratuvarında
“16S rRNA analizi”;
standart identifikasyon
yöntemlerine alternatif olarak değil,
bu yöntemlerle beraber kullanılmalı,
sonuçları dikkatle yorumlanmalı ve
özellikle alışılmadık türlerin
bildirimi dikkatle yapılmalıdır!**



Teşekkür Ederim

